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THE ISOLATION AND CHARACTERIZATION OF PSEUDOMONAS HALODURANS, SP NOV SELECTED FROM THE GREAT BAY ESTUARY AS A HALOTOLERANT SPECIES

ARTHUR ROSENBERG

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THE ISOLATION AND CHARACTERIZATION OF *PSEUDOMONAS HALODURANS*, SP. NOV. SELECTED FROM THE GREAT BAY ESTUARY AS A HALOTOLERANT SPECIES

by

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To my loving and understanding wife, without whom I could not have endured the trials and tribulations of writing this thesis.

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ABSTRACT

THE ISOLATION AND CHARACTERIZATION OF PSEUDOMONAS HALODURANS, SP. NOV. SELECTED FROM THE GREAT BAY ESTUARY AS A HALOTOLERANT SPECIES

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ARTHUR ROSENBERG

The bacterial population of the Great Bay estuarine complex, Durham, New Hampshire, was investigated for tolerance to NaCl additions up to 3.05 M. Using a modified 2216E (0.1 % peptone and yeast extract) agar medium adjusted to a salinity of 26 $\pm$ 1 ppt as a standard, the bacterial CFU/ml were recorded after incubation for two weeks at 20 C. When 3-fold NaCl (about 1.0 M) was added to the agar medium, less than 20 % of the colonies on the standard medium developed. When the modified 2216E medium contained more than 2.0 M NaCl, the CFU/ml were less than 1 % of the standard salinity plates. The colonies appeared to be similar, as beige 1-2 mm in diameter, smooth, circular, raised to convex, and entire colony types. Bacterial cells isolated directly from these salt-supplemented plates were gram-negative, nonmotile bacilli, measuring 2.0 to 2.5 µm in diameter by 3.5 to 5.0 µm in length. By colonial appearance, sugar utilization, antibiotic sensitivity, catalase and oxidase reaction, DNA-DNA hybridization, and other taxonomic determinations conducted at 26 ppt and the higher salinities; these halotolerant bacterial isolates were always similar and identical.

The halotolerant bacterial cells did not liquify gelatin, had a temperature range between 5 and 35 C with an optimum temperature between 20 and 25 C, and a guanine plus cytosine (GC) molar % of 63.2 $\pm$ 0.5. These and other diagnostic characteristics placed the bacterium in the genus Pseudomonas. The ability of these cells to tolerate supplemental NaCl greater than 2.0 M suggested the species name halodurans sp. nov., meaning halotolerant.

P. halodurans grown in modified 2216E medium were 0.5 to 0.7 µm in diameter and 1.5 to 1.7 µm in length. The cells were arranged singly or in pairs, and motile with a single polar flagellum. Flagellation occurred only in saline medium up to 0.9 M NaCl. The salt stress of P. halodurans was not NaCl specific since other salts affected the growth response similarly. The requirements for major ions on a Na deficient 0.1 % glucose synthetic seawater medium were: Na above 35 mM, Mg above 8 mM, K above 4 mM, and Ca above 3 mM. P. halodurans grew optimally at ionic concentrations representative of the estuary.

The growth of P. halodurans was inhibited progressively as NaCl was supplemented to media to a final tolerance of 3.45 M. This salt inhibition was ionic rather than osmotic since glycerol and sucrose at similar osmotic concentrations did not cause inhibition. The bacterium was not adaptive to varying salt concentrations indicating its stable stenohaline character. Cells in late lag growth were more resilient to NaCl stress than at other stages of growth.

Respiration was affected similarly to growth by varying NaCl concentrations. As indicated by triphenyl tetrazolium chloride reduction, the cells were respiratory sufficient up to 3.45 M NaCl. Tests with methylene blue and dichloroindophenol reduction indicated NaCl stress affected respiration at or before the flavoprotein-quinone but not the cytochrome electron transport system. The regulatory enzyme, aspartate transcarbamylase, indicated optimum activity of NaCl concentrations representative of the estuary.

Accumulation of K by the cells during NaCl stress was a compensatory mechanism which was related to increasing cell size. Cell envelopes accumulated ^{22}Na during $^{22}NaCl$ stress.

DNA, RNA, protein, carbohydrate, and phospholipid ratios did not vary in the cells during NaCl stress. Likewise, total protein, amino acids, and phospholipid did not vary in the cell envelopes of NaCl-stressed cells.

P. halodurans was a halotolerant bacterium selected from the Great Bay estuary with high resistance to supplemental NaCl. The persistent stenohaline nature of the bacterium indicated a dynamic physiological adaptation to NaCl stress rather than genetic or biochemical alteration.

CHAPTER I

INTRODUCTION

Halotolerance, the ability to tolerate NaCl concentrations many times greater than those encountered by an organism in its natural environment, is more common among terrestrial than marine bacteria (MacLeod, 1965). Since terrestrial bacteria arose probably from marine bacteria (MacLeod, 1965), the possibility exists that halotolerance occurs in the marine environment. However, the most detailed studies of marine bacteria deal with their requirements for, rather than with their tolerance of, NaCl (MacLeod, 1965 and 1968). NaCl concentrations a few times higher than those of seawater reportedly inhibit or arrest growth of most marine bacteria.

The present study was initiated with an investigation of the eury-or stenohaline response of the estuarine bacterial population to the selective effects of the various major ions of seawater in concentrations greater than those of seawater. The Great Bay estuarine complex was chosen as estuarine bacteria were suspected of tolerance to fluctuations in salinity. Would supplemental major ions, especially NaCl, exert toxic effects on the entire estuarine bacterial population or would halotolerant bacterial species emerge? If so, would diversified bacterial species be selected, or would supplemental NaCl concentrations select for a halotolerant bacterial isolate? The remainder of the

investigation deals with the basis of the halotolerance of selected bacteria. How would supplemental NaCl affect the physiology of halotolerant isolates and was halotolerance adaptable? Was the effect ionic or osmotic? Did the intracellular ionic concentration increase as the supplemental medium NaCl increase or were the ions excluded? What was the effect of an increase in the intracellular ionic concentration on the biosynthetic and enzymatic machinery of the cells? Investigations of these and other questions may contribute to a fuller understanding of halophilism and halotolerance.

CHAPTER II

LITERATURE REVIEW

Marine bacteria have been defined by ZoBell and Upham (1944) as those bacteria isolated from the sea which upon initial isolation required seawater for their propagation. This definition of marine bacteria has been accepted generally but has raised questions. How stable is the seawater requirement? What functions do the ions dissolved in seawater serve for the microorganisms indigenous to it? How closely restricted are marine bacteria to the salinity of the ocean? What are the overall effects of the salts dissolved in seawater upon marine bacteria? A number of investigations have contributed to answering these fundamental questions regarding marine bacteria as summarized by MacLeod (1968).

The salinity of seawater has been defined as the weight in g of the dissolved inorganic matter in 1 kg of seawater after all Br and I have been replaced by the equivalent amount of Cl, all carbonate converted to oxide, and all organic matter removed (Knudsen, 1901). The total salt content of seawater is about 0.45 % greater than its salinity if defined in this way. The factors affecting the salinity of the open ocean are basically climatic ones. The salinity distribution in the open ocean tends to be zonal and range from 32 to 37.5 ppt. The most abundant ion in seawater of a salinity of 35 ppt is Cl with a molarity of 0.5483 (Sillén, 1961). The concentration of Na, the second most

abundant ion in seawater, is 0.4702 M (Sillén, 1961). Sodium chloride constitutes 85 % of the total salinity of the open ocean. The oceans of the world are not saturated with NaCl as the solubility of this salt in water ranges from 0 to 6 M (Hodgman, 1959). It is not surprising that NaCl and the other ionic species dissolved in the oceans of the world exert a profound influence on life.

Bacteria are capable of growing in natural environments containing the full range of NaCl concentration (Brown, 1964). Only a few living creatures can survive in both fresh water and seawater. This is true for bacteria, fungi, green plants, and animals. In the case of bacteria, cultural conditions such as media (Kluyver and Baars, 1932; Larsen, 1962), nutrient concentrations in the medium (Ingram, 1957), and incubation temperature (Novitsky and Kushner, 1975) altered significantly an organism's response to NaCl. A classification of microorganisms on the basis of their reaction to NaCl follows:

Description	Microbial Reaction
Halophobic bacteria	Grow only in media containing less than 0.2 M NaCl.
Facultative halophilic bacteria	Grow in media containing 0.2 - 4.3 M NaCl, but do not require NaCl for growth.
Obligate halophilic bacteria Slight halophilic bacteria	Grow optimally in media containing 0.5 - 3.0 M NaCl.
Moderate halophilic bacteria	Grow optimally in media containing 0.5 - 3.0 M NaCl, but grow in media containing up to 4.5 M NaCl.
Extreme halophilic bacteria	Grow optimally in media containing 2.6 - 5.8 M NaCl and do not grow below 2.0 M NaCl.

This rough classification is based upon convenience. There is a problem in choosing specific threshold concentrations of NaCl in order to make exact distinctions in terms and intermediate types (Schoop, 1934; Flannery, 1956; Ingram, 1957; Larsen, 1962).

The highest bacterial plate counts from seawater samples were obtained if the medium was prepared with seawater or 0.5 M NaCl (Fischer,1894). The necessity for seawater or NaCl was presumed to reflect a requirement of marine bacteria for a medium in which salts maintained a suitable osmotic pressure. This conclusion originated from observations made by Harvey (1915) that marine bacteria lysed when suspended in seawater too greatly diluted with distilled water. However, Richter (1928) demonstrated the requirement of a marine luminous bacterium was satisfied only by specific Na salts. This observation has been amplified by MacLeod and Onofrey (1956) who demonstrated that for the marine bacteria investigated, Na (as well as K and Mg) was required for growth. These observations suggested that marine bacteria were distinguishable from terrestrial forms by a demonstrable need for Na in the medium for growth. The marine bacterium originally designated as B-16 (MacLeod and Onofrey, 1956) and now classified as Alteromonas marinopraesens (Baumann et al., 1972) required 0.2 - 0.3 M NaCl for optimal growth (MacLeod and Onofrey, 1957a,b, 1958). Below these concentrations of Na, the amount of growth obtained in 48 h was roughly proportional to the concentration of Na. A. marinopraesens

did not grow at Na concentrations significantly below 0.03 M even after attempts at adaptation. The requirement for Na was not replaced by Li, Rb, K, and Cs except for short incubation periods (MacLeod and Onofrey, 1956).

Tyler et al. (1960) examined 96 bacteria isolated from seawater in Florida. They were gram-negative rods or spiral forms, and though a peptone medium (0.1 % in distilled water) was used, the cultures required added NaCl for good growth. They concluded that an irreplaceable minimal Na requirement was a distinctive property of marine bacteria.

Minimal sparing action for Na has been demonstrated by K or Mg (Pratt, 1974; Hidaka and Sakai, 1970; MacLeod, 1965). MacLeod and Onofrey (1957a)using A. marinopraesens demonstrated a minimal sparing action for Na by K and Mg at NaCl concentrations greater than 0.01 M. K and Mg concentrations 2 - 4 times greater than seawater concentrations spared up to 20 % of the Na requirement. Pratt (1963) and Pratt and Austin (1963) demonstrated that the marine bacteria investigated, exhibited up to 40 % replacement of Na by K and Mg after the minimal Na requirement (0.01 M) was satisfied. This observation was amplified by Tedder (1966) who examined 20 marine isolates in a chemically defined medium. Using 0.02 M Na as a suboptimal amount, she found that the addition of K ranging from 0.1 to 0.3 M increased the growth rate from 5 to 100 % compared to that produced by the optimal Na concentration (0.2 to 0.5 M) and 0.008 M K. These observations suggested that in the marine bacteria tested, there was a nonspecific

Na requirement that can be spared differentially by nonspecific solutes such as K and Mg. The irreplaceable minimal Na requirement suggested a specific function for Na in marine bacteria (MacLeod, 1968).

The response of three marine bacteria to anions indicated that Cl produced optimal growth compared to SO_4, NO_3, or I (MacLeod and Onofrey, 1957b). Cl and Br were used interchangeably by these bacteria. The effects of the halides and Na on both rate and extent of growth were so similar that the function of halides and Na were considered closely related in the metabolism of marine bacteria (MacLeod and Onofrey, 1957a,b). The investigations on gram-negative bacteria isolated from the marine environment suggested a specific Na requirement and it was concluded that Na salts have little, if any, osmotic functions (MacLeod, 1965, 1968).

Moderate halophilic bacteria isolated from spoiled fish (Flannery, 1956; Ingram, 1957), curing brines (Dussault, 1955; Keller and Henis, 1967), and salted food products (Scott, 1957; Larsen, 1962) demonstrated a specific Na requirement for growth. Robinson and Gibbons (1952) found that NaCl could not be replaced by specific Na salts, or by Li, K, Mg, or NH_4 salts in growth experiments on Micrococcus halodenitrificans. Christian (1956) extended the investigations on M. halodenitrificans and found that KCl substituted for 75 % of the Na requirement on a molar basis. He also demonstrated that Vibrio costicolus had a specific, nonsubstitutable Na requirement for growth. Lindeberg (1958) noted

that a moderate halophilic bacterium, Achromobacter sp., isolated from salted herring brine had a specific Na requirement that was not replaced by K, Li, or NH_4. The response of the bacterium to anions indicated that Cl produced optimal growth compared to Br, SO_4, and HPO_4. Thus, moderate halophilic bacteria demonstrated a specific Na requirement for growth.

If seawater was evaporated by the sun, solar (sea) salt was produced (Baas-Becking, 1931). The solar salt was populated frequently by extreme halophilic bacteria which originated in the marine salterns (Petrowa, 1933; Dussault, 1933). Extreme halophilic bacteria have been demonstrated in the Great Salt Lake (Smith and ZoBell, 1937; ZoBell et al., 1937), the Dead Sea (Elazari-Volcani, 1940), and spoiled fish, bacon and hides preserved in (presumably) solar salt originating from seawater (Flannery, 1956; Ingram, 1957; Ishida and Fujii, 1970). Extreme halophilic bacteria grew in media containing at least 2.0 M NaCl (Larsen, 1967). Growth of extreme halophilic bacteria in medium devoid of Na and Cl did not occur (Schoop, 1935; Hess, 1942; Weber, 1949; Larsen, 1962).

In experiments attempting partial cation substitution on extreme halophilic bacteria, Brown and Gibbons (1955) using Halobacterium salinarium and Christian (1956) using H. halobium, obtained evidence, that on a molar basis, about 70 % of the amount of Na required was replaced by K. KCl was the only salt among a number tested which gave definite support to growth besides NaCl. However, Elazari-Volcani (1940, 1943,

1944) indicated that for growth of Halobacterium sp., Mg substituted for Na during short incubation periods. The Halobacterium sp., isolated from the Dead Sea waters which contain approximately 1.5 M $MgCl_2$ and 1.2 M NaCl (Brock, 1969) did not grow unless the medium (harboring 0.5 - 0.8 M $MgCl_2$) contained at least 2.6 M NaCl. These results suggested that in their natural habitat, these bacteria made use of Mg to replace in part the function played by Na (Elazari-Volcani, 1944). However, extreme halophilic bacteria demonstrated a specific requirement for Na.

The literature on the specific salt requirements of halophilic bacteria, indicated the paucity of information in this area. Some halophilic bacteria were specific in their requirement for both Na and Cl. Others had a specific requirement for Na and were more or less unspecific in their requirement for anions, whereas still others were more or less unspecific in their requirement for cations and anions. A specific requirement for Na and/or Cl suggested a nutritional function; whereas a nonspecific requirement implied an osmoregulatory function (Richter, 1928). These differences in ion requirements by different bacterial cultures suggested that halophilism was not concerned with one single biochemical property common to halophilic bacteria. Thus, the cause of the salt requirement may have been quite different in halophilic bacteria (Larsen, 1962). Furthermore, in the same bacterium, NaCl may have been required for more than one function.

Since bacteria are distributed ubiquitously in the biosphere, it is of interest to know if the requirement of Na for growth was unique for bacteria from the marine environment (MacLeod, 1968). Both moderate and extreme halophilic bacteria have been isolated from soil and fresh water sources (Hof, 1935; Stuart, 1938; Larsen, 1962). Among nonhalophilic bacteria, two strains of Rhodopseudomonas studied in a chemically defined medium required 0.002 M Na for growth (Sistrom, 1960). Bacteroides succinogenes, an organism isolated from the rumen of a steer, required 0.01 M Na for growth in a defined medium (Bryant et al., 1959). Goldman et al. (1963) demonstrated a definitive requirement for Na (0.02 M) for growth in a number of lactic acid bacteria isolated from meat-curing brines. These confirmed reports raised the question: Is the Na requirement more widespread among bacteria than commonly supposed? An affirmative answer might supplement speculations on the origin and rise of halophilism.

In the work on halophilic bacteria, interest has been expressed in the constancy of the halophilic character (Pratt, 1974; Larsen, 1962). Do halophilic bacteria change quantitatively, or even abandon, their requirement for NaCl? Marine bacteria that required seawater in the medium on initial isolation developed a capacity to grow in media prepared with fresh water after cultivation for some time in the laboratory (ZoBell and Michener, 1938; ZoBell, 1946). In contrast, Pratt and Waddell (1959) failed to adapt marine bacteria to media

prepared with artificial seawater diluted 10-20 fold with fresh water. When thick suspensions of these marine bacteria were plated on nutrient media prepared with artificial seawater devoid of NaCl, few colonies were obtained. These colonies were considered mutants which no longer required Na for growth. MacLeod and Onofrey (1963) after serially streaking plates of trypticase medium containing progressively lower concentrations of Na with A. marinopraesens observed that the bacterium eventually was "trained" to grow on the medium prepared without added Na. When tested in a chemically defined medium devoid of NaCl, no growth occurred. This observation agreed with earlier data by MacLeod and Onofrey (1956, 1957a, b), Stanier (1941), and Littlewood and Postgate (1957) who reported failure to train marine bacteria to grow in media devoid of NaCl. MacLeod and Onofrey (1963) concluded that the growth of certain marine bacteria in the trypticase medium devoid of NaCl was due to the presence of 0.028 M Na (as determined by flame photometry) present as a contaminant. Thus, growth at appreciably reduced Na concentrations was induced and maintained only if the medium was sufficiently complex (MacLeod and Onofrey, 1963). When tested in chemically defined media devoid of Na (contamination of Na was less than 5×10^{-5} M), bacteria isolated initially from the marine environment demonstrated a stable Na requirement.

The constancy of the halophilic character of bacteria can also be investigated by studying the response of marine bacteria to supplemental NaCl concentrations in excess of

those amounts found in the environment. Marine bacteria, since they live in the sea, must be NaCl-tolerant organisms (MacLeod, 1965). Seawater contains approximately 0.47 M Na (Sillén, 1961). Three marine bacteria investigated by MacLeod and Onofrey (1957a) were inhibited by the presence of 0.8 M NaCl. Of 15 marine bacteria examined by Tyler et al. (1960), all grew at 0.8 M NaCl, 9 grew at 1.4 M NaCl, and none grew at 2.6 M NaCl. ZoBell (1946) noted that very few marine bacteria grew in seawater with 2.0 M NaCl added, and no growth occurred in seawater with 4.0 M NaCl added. Similar results to those of ZoBell were obtained by Brown and Turner (1963) studying 12 marine bacteria. In contrast, marine bacteria have been reported that tolerated up to 4.5 M NaCl (Shah and deSa, 1964; Forsyth et al., 1971). Although extreme halotolerant bacteria do exist in the marine environment, optimal growth occurred at NaCl concentrations similar to those of the environment indicating the stenohaline response of marine bacteria (MacLeod, 1965, 1968).

The ability to tolerate or require elevated NaCl concentrations was exhibited in parasitic and branching, filamentous bacteria. Bell and Latham (1975) observed a parasitic marine Bdellovibrio sp. required 0.25 to 0.7 M NaCl for optimal growth. Growth of Bdellovibrio was stimulated by 0.002 M $CaCl_2$ and $MgCl_2$ added to the NaCl supplemented-yeast extract, peptone medium. Gochnauer et al. (1975) isolated a filamentous actinomycete (Actinopolyspora halophila gen. et sp. nov.) from solar salt. The organism has a mucopeptide

layer, required 3.3 to 5.0 M NaCl for optimal growth, and did not grow below 2.0 M NaCl. Thus, A. halophila was an extreme halophile, the first known in the family Nocardiaceae. Tresner et al. (1968) studied the NaCl tolerance of 1300 Streptomyces strains (containing 313 different species). Only 4 % of the Streptomyces were halotolerant species capable of surviving in 1.0 to 2.7 M NaCl. Approximately 50 % of the Streptomyces strains tolerated up to 1.0 M NaCl; the remainder did not tolerate 0.3 M NaCl. Actinomycetes have been isolated infrequently from the sea (Mayers et al., 1960). The ability to tolerate and/or require NaCl concentrations greater than those found in the marine environment thus occurs in a wide diversity of bacteria.

Stuart and James (1938) claimed to have adapted extreme halophilic bacteria of the Sarcina-Micrococcus type (now classified as Halococcus, Kocur and Hodgkiss, 1973), which had a minimum NaCl requirement of 0.8 - 1.6 M, to grow in the absence of NaCl, provided the cultures were older than 30 days. Hess (1942), studying these same Halococci as well as Halobacterium sp., could not reproduce the findings of Stuart and James. All subsequent attempts to adapt moderate and extreme halophilic bacteria to grow in less than 0.7 and 2.0 M NaCl, respectively, have been unsuccessful (Christian, 1956; Ingram, 1957; Kushner, 1964;Limsong and Frazier, 1966; Larsen, 1967, 1973). Where transformations of salt tolerant and salt requirement characteristics have been reported (Baars, 1930; Kluyver and Baars, 1932; Petrowa, 1933; Hof , 1935;

Stuart and James, 1938; Shewan, 1938), the purity of the culture has been questioned (Ingram, 1957; Littlewood and Postgate, 1957).

Several investigations have been made to determine the NaCl concentration which arrested growth of the various types of nonhalophilic bacteria (Larsen, 1962). Work with pure cultures has shown that many nonhalophilic bacteria have a fixed tolerance to NaCl (Ingram, 1957). Hof (1935) and Doudoroff (1940) failed to alter the salt tolerance of Escherichia coli significantly. Christian (1955) using Salmonella oranienberg and Burick (1950) using Serratia marcescens failed to "train" these bacteria to tolerate greater than 0.5 M NaCl. Where a transformation in the salt tolerance has been reported for nonhalophilic bacteria (Anderson, 1954; Foda and Vaughn, 1950), the purity of the culture was questioned again (Ingram, 1957).

NaCl tolerance of nonhalophilic bacteria varies considerably from species to species and even for different strains of the same species (Scott, 1957). In general, the obligate anaerobic spore-formers (i.e. Clostridium sp.) and terrestrial gram-negative rods were most sensitive to NaCl, many of these being completely inhibited at 0.5 - 0.8 M NaCl (Hill and White, 1929; Larsen, 1962). Likewise, 0.5 - 0.8 M NaCl inhibited most marine species as well (MacLeod, 1965). Aerobic spore-forming bacteria (i.e. Bacillus sp.) were usually quite NaCl tolerant, growing at concentrations up to 2.6 - 3.5 M NaCl (West et al., 1941). The most tolerant of

the nonhalophilic bacteria were the micrococci; many strains grew slowly in the presence of 4.3 M NaCl (Christian, 1955, 1956). This latter observation was not surprising since the micrococci were closely related to the extreme halophilic cocci, Halococcus morrhuae and H. litoralis (Larsen, 1967). Although bacteria existed that did not require NaCl for growth, these organisms demonstrated a considerable tolerance to NaCl, similar to the tolerance demonstrated by some marine bacteria. Is there a common denominator that can, at least in part, explain NaCl tolerance by bacteria?

The requirement of moderate and extreme halophilic bacteria for a high concentration of NaCl for growth led to an interest in the actual intracellular concentrations of Na and K in these bacteria (Larsen, 1962). An early theory on the existence of moderate and extreme halophilic bacteria was that they excluded NaCl from the cell and maintained a relatively dilute protoplasm (Robinson et al., 1952). Experimental evidence has accumulated demonstrating that the intracellular Na concentration was quite high and reached concentrations approaching those of the growth medium (Gibbons and Baxter, 1953; Christian, 1956; Christian and Ingram, 1959 a,b; Holmes, 1964; Masui and Wada, 1973). K was accumulated intracellularly close to its solubility limit (Brown and Gibbons, 1955; Christian, 1956; Masui and Wada, 1973). This latter observation was amplified by Christian and Waltho (1961, 1962) after testing a number of facultative halophilic, marine, moderate and extreme halophilic bacteria and their response to NaCl.

These investigators noted a relation between the intracellular K concentration and the tolerance of these bacteria to NaCl. The higher the K content of the cells, the better the tolerance to NaCl. Christian and Waltho (1962) suggested that the high K content of the facultative halophilic bacteria might impart a resistance to plasmolysis and dehydration, thus overcoming the detrimental effects of the medium salts. The Na and K content of marine, moderate and extreme halophilic bacteria approached that of the growth medium (Christian and Waltho, 1962; Larsen, 1962) and suggested that Na and K might play important roles in regulating the metabolism of these bacteria (Brown, 1964; Ginzburg et al., 1970; Gouchnauer and Kushner, 1971).

The conclusions concerning the relations between medium and intracellular Na and K must be regarded with caution. Many of the intracellular Na and K values were determined at one concentration of NaCl and KCl and/or at one stage of growth (normally the late logarithmic phase). Thus, it was not entirely possible to conclude to what extent the intracellular Na concentration was dependent on the medium Na concentration. Medium Na concentrations and the age of the culture influenced significantly the intracellular concentrations of Na and K (Schultz and Solomon, 1961; Masui and Wada, 1973). Additionally, methods to determine intracellular Na and K values must be improved and standardized before data can be interpreted meaningfully and reliably.

The observation that most marine bacteria lysed when placed in distilled water or in solutions suitably hypotonic

to seawater (Harvey, 1915; Hill, 1929; Pratt and Riley, 1955; Tyler et al., 1960; MacLeod and Matula, 1961, 1962; Buckmire and MacLeod, 1965; DeVoe and Oginsky, 1969a, b) led early investigators to conclude that salts were required to prevent autolysis by maintaining a suitable osmotic pressure (Harvey, 1915; Hill, 1929). However, Pratt and Riley (1955) demonstrated that different salts differed in their capacity to prevent lysis. For a number of marine bacteria, NaCl and LiCl were about twice as effective as KCl and NH_4Cl in preventing lysis. This observation was confirmed and amplified by MacLeod and Matula (1961, 1962) who observed that for all marine bacteria investigated, divalent cations (especially Mg and Ca) were 10 to 50-fold more effective than monovalent cations (Na, Li, K, or NH_4) in preventing disruption of the cells. The order of effectiveness to prevent lysis of the divalent cations was similar to their abundance in seawater and their capacity to form chelate complexes. Even more effective than divalent cations was 0.0005 M spermine (Mager, 1959a, b). If the effect of the compounds had been due to their osmotic activities, they should have been equally effective at the same total ionic or molecular concentration (Buckmire and MacLeod, 1965).

Further evidence that NaCl did not prevent lysis of marine bacteria by osmotic action arose when the intracellular Na and Cl concentrations of A. marinopraesens were measured at medium NaCl concentrations ranging from 0.055 to 1.022 M (Takacs et al., 1964). Within experimental error, these

investigators found that the intracellular Na and Cl concentrations were the same as that of the medium. These findings suggested that Na and Cl were freely exchangeable across the cell membrane and that no gradient of these two ions was maintained between the inside and outside of the cells. NaCl thus, did not prevent lysis of the cells through osmotic action (Buckmire and MacLeod, 1965; MacLeod, 1965, 1968). Intracellular K concentrations, in contrast, were approximately double the medium K concentration (0.009 to 0.014 M). Recently, Matula et al. (1970) studying A. marinopraesens demonstrated intracellular K concentrations 12 and 17-fold greater than the medium K (0.015 M) in the presence of 0.22 and 0.61 M medium NaCl, respectively.

The lysis of marine bacterial cells at low NaCl concentrations (less than 0.1 M) took place in two stages (MacLeod and Matula, 1962). The first stage was a rapid autolysis of the cells whose cell walls were weakened by the Na deficit (Pratt, 1974). Buckmire and MacLeod (1965) and Drapeau and MacLeod (1965) analyzed the effects of different salts (NaCl, LiCl, KCl, and NH_4Cl) and their capacity to prevent turbidity changes in suspensions of whole cells and cell envelopes. They observed that NaCl and LiCl were twice as effective as NH_4Cl and KCl in preventing turbidity changes in whole cell suspensions whereas the four salts were equally effective in preventing turbidity changes in cell envelope suspensions. Thus, the differential effect of these salts on the turbidity of whole cell suspensions was due to the

differential capacity of the salts to prevent leakage of low molecular weight intracellular organic solutes from the cells.

The second stage of lysis was a slower continued disintegration of cell envelopes (MacLeod, 1968; Pratt, 1974). The disintegration of cell envelopes was thought to be the result of the effects of ionic strength and particularly divalent cations on the conformation of membrane proteins. Proteolytic autolysis was considered to be a direct consequence of such conformational changes (Brown, 1960, 1961,1962). However, Buckmire and MacLeod (1965) did not favor this hypothesis, because the lysis of whole cells was such a rapid process that it seemed unlikely that it was due to the action of an enzyme. These investigators demonstrated that the effect of heat (100 C for 15 min) and NaCl (0.01 - 0.1 M) on the release of the hexosamine-containing fraction of the cell envelopes was analogous to the effects of heat and salts on the denaturation of a polyanion and was explainable in terms of a polyelectrolyte theory (Kotin, 1963). This suggested two conclusions: (1) since heated envelopes released just as much hexosamine-containing material as unheated envelopes, cell envelope disintegration was not enzymatic; and (2) the disintegration of the cell envelopes, confirmed with electron micrographs, suggested that the cell envelope was composed of a series of subunits. Subsequent analyses have determined that the subunits contained a large number of negatively charged groups, free phosphates of the phospholipids and terminal carboxyl groups associated with amino acids (Forsberg

et al., 1970a, b; Unemoto et al., 1973; Costerton et al., 1974). Na screened these free electronegative charges, thereby stabilizing the cell envelope, especially the mucopeptide layer (Buckmire and MacLeod, 1965; DeVoe and Oginsky, 1969a). Mg was more effective than Na in maintaining envelope integrity since it formed divalent ionic bridges (chelate) between the negatively charged groups (Rayman et al., 1967; McClare, 1967; DeVoe and Oginsky, 1969b; D'Aoust and Kushner, 1971; Rayman and MacLeod, 1975). The ability of KCl to prevent lysis of marine bacteria was due to its capacity to balance the internal osmotic pressure of the cells when exposed to a hypotonic solution (Unemoto et al., 1973). It may be concluded that lowering the salt concentration destroys the continuity of the mucopeptide layer of marine bacteria thereby weakening the cell envelope to the point where the intracellular osmotic pressure caused the envelope to rupture (MacLeod, 1968).

In the past, the lytic phenomenon of moderate halophilic bacteria has been ascribed to osmotic effects (Larsen, 1962). However, Christian and Ingram (1959 a,b) demonstrated that, similar to marine organisms, V. costicolus, V. metschnikovii, and V. ichthyodermis lysed at higher concentrations of KCl and NH_4Cl than with NaCl or LiCl. The data suggested that in hypotonic solutions the smaller hydrated ionic radii (K and NH_4) penetrated the cell envelope causing increased internal osmotic pressure. The subsequent swelling deformed and finally ruptured the cell envelopes weakened by the Na deficit.

Takahashi and Gibbons (1959) found that the susceptibility of M. halodenitrificans to lysis in hypotonic solutions was increased by growth in a medium containing less than 0.6 M NaCl. They noted that this susceptibility was related to the decrease content of a characteristic cell envelope component, diaminopimelic acid. These investigators concluded that NaCl might be required for cell envelope synthesis and integrity. If the lytic capacity of NaCl, LiCl, NH_4Cl, and KCl on moderate halophilic bacteria was the same for marine bacteria, the mode of action might be explained by the hydrated ionic radii theory of Christian (1956).

The lysis of extreme halophilic bacteria also was assumed to be due solely to osmotic effects (Larsen, 1962). However, Abram and Gibbons (1960, 1961) showed that, similar to marine and moderate halophilic bacteria, NaCl and LiCl were more effective than KCl and NH_4Cl in preventing lysis of H. cutirubrum and H. halobium and suggested strongly that Christian's theory was applicable to halophilic bacteria in general. They observed that H. cutirubrum and H. halobium lysed at a fixed NaCl concentration, independent of that at which the cells were grown. On the basis of this and further observations on morphological alterations, these investigators suggested that the presence of NaCl concentrations greater than 2.0 M NaCl were needed to screen the electro-negative charges of the cell envelope and maintain the rod-shape of the bacteria. Subsequent investigations have shown that the dependence upon high concentrations of Na and Mg (0.5 M $MgCl_2$

or greater) by halobacteria to maintain cell envelope integrity was due primarily to three factors: (1) the unique presence of long chain alkyl groups joined by ether (not ester) linkages to glycerol (Smithies et al., 1955; Sehgal et al., 1962; Kates et al., 1963, 1965; Kushner et al., 1964; Brown, 1965); (2) the presence of 3 times more acidic than basic amino acids (Baxter, 1959; Brown, 1963, 1964; Brown and Shorey, 1963; Kushner et al., 1964; Kushner, 1964; Kushner and Onishi, 1966; Onishi and Kushner, 1966; Stoeckenius and Rowan, 1967; Steenland and Larsen, 1969; Reistad, 1970); and (3) the absence of a mucopeptide "rigid layer" (muramic acid and diaminopimelic acid absence) (Brown and Shorey, 1963; Mohr and Larsen, 1963; Salton, 1964; Kushner et al., 1964; Larsen, 1967; Soo-Hoo and Brown, 1967; Marshall et al., 1969; Mescher et al., 1974).

Among gram-negative bacteria, there existed a spectrum of susceptibility to lysis ranging from bacteria which required high salt concentration (2.0 M NaCl) to prevent disruption of the cells to those which maintained their integrity in distilled water (MacLeod, 1965). Bacteria most susceptible to lysis were the extreme halophilic bacteria, followed by the moderate halophilic bacteria, and at the lower end of the spectrum, the marine bacteria. Organisms of terrestrial origin were considered generally not to be susceptible to lysis (MacLeod, 1965). However, two nonmarine species, Franciscella tularensis and Neisseria perflava demonstrated intracellular solute leakage and decreased cell envelope

integrity after brief exposure to distilled water (Mager, 1959a, b). From the data concerning lysis, two general conclusions were drawn: (1) as more marine and nonmarine bacteria are studied, clear-cut distinctions between marine and nonmarine species of bacteria cannot be made solely on the basis of lytic and susceptibility; and (2) marine bacteria were the beginning of a series of organisms with increasing Na and/or Cl requirements.

The obligate growth requirement of marine bacteria for Na cannot be ascribed entirely to maintaining cell envelope integrity. Indeed, the specific requirement for growth may be more a function of the specific Na requirement of marine bacteria for metabolism (MacLeod and Onofrey, 1957a; MacLeod et al., 1958; Payne, 1960; Tyler et al., 1960). Na and K in the medium have been shown to affect markedly both the rate and extent of growth of marine bacteria examined (MacLeod, 1965, 1968). However, in all cases tested, concentrations of Na required for a maximal rate of substrate oxidation by cell suspensions inhibited the activity of cell-extracted enzymes involved in these oxidation processes (MacLeod et al., 1958; MacLeod and Hori, 1960; MacLeod et al., 1960; Pratt and Happold, 1960). Utilizing A. marinopraesens and various mono-, di-, and tri-basic acids as well as a nonmetabolizable analog (α-amino-isobutyric acid-1-^{14}C), Drapeau and MacLeod (1963) and Drapeau et al. (1966) demonstrated an obligate requirement of both Na and K for the transport of exogenous substrates into the cells. In addition,

the amount of Na, but not K, required for the maximum rate of oxidation varied with the substrate being oxidized. These studies, in conjunction with kinetic analysis of the Na-dependent transport process (Wong et al., 1969) suggested that the prime rule for Na was to increase the affinity of a carrier or binding protein for the molecule to be transported (Thompson and MacLeod, 1971). This theory contradicted an earlier conclusion by Payne (1958, 1960) and Rhodes and Payne (1962, 1967) that Na was involved in the formation (induction) of the transport ("permease") system.

Thompson and MacLeod (1971, 1973) concluded that K was needed to accumulate and increase the rate of uptake of substrate. Since K accumulation has been demonstrated in terrestrial bacteria (Larsen, 1962), a transport system similar to that of marine bacteria may be common. MacLeod (1968) speculated about a Na-, K-activated ATPase mechanism of active transport in marine bacteria, similar to that seen in animal cells (Crane, 1965; Kipnis and Parrish, 1965; Schultz and Curran, 1970). However, no such mechanism has yet been demonstrated in marine bacteria (MacLeod, 1968).

Clear evidence is available that intracellular enzyme systems of moderate and extreme halophilic bacteria were active in NaCl concentrations ranging from 0.4 to 5.8 M, although optimal activity of cell-free enzyme preparations was not always at similar NaCl concentrations as that necessary for optimal growth of the organism (Robinson et al., 1952; Baxter and Gibbons, 1954, 1956, 1957; Larsen, 1962, 1967;

Kushner, 1964). However, very little evidence was available to demonstrate a Na-dependent transport system in moderate and extreme halophilic bacteria (Larsen, 1962; Kushner, 1968). A singular report by Stevenson (1966) indicated that the uptake of glutamate by H. salinarium was dependent specifically on a NaCl concentration greater than 3.0 M. Additionally, KCl which was present in concentrations approaching saturation (4.5 M) within the cells, activated the enzymes studied by Stevenson (1966). Stevenson found that the uptake of glutamate by H. salinarium was by active transport. In light of the dependence on Na and K gradients between the inside of animal cells and the medium for ATPase activity associated with active transport (Kleinzeller and Kotyk, 1961), it is attractive to think of an ATPase system operational in moderate and extreme halophilic bacteria. Unfortunately, no such system has yet been found (Kushner, 1968).

The evidence thus far suggests that Na and K have specific functions in marine bacteria in transport mechanisms. Many more investigations on metabolic systems of many other obligate halophilic bacteria must be performed before general conclusions regarding the specific requirement for Na for substrate uptake and metabolism can be drawn.

Microorganisms other than bacteria tolerated or required various concentrations of NaCl (Larsen, 1962). Many of these organisms grew optimally at NaCl concentration indigenous to their environment, while other organisms grow optimally at lower NaCl concentrations than their environment

(Elazari-Volcani, 1940; Brock, 1969). The ability to tolerate or require NaCl concentrations greater than that in seawater was demonstrated in protozoans, ciliates, and shrimp (Larsen, 1962). Elazari-Volcani (1940, 1943) demonstrated that Dimastigamoeba sp. and Zoomastigina (Calonympha) isolated from the Dead Sea (1.2 M NaCl, 1.5 M $MgCl_2$, Brock, 1969) grew optimally between 1.0 and 2.5 M NaCl. Loefer (1939) observed adaptation of fresh water ciliates (Colpidium) and flagellates (Euglena) to NaCl concentrations ranging from 0.5 to 1.5 M. Flowers and Evans (1966) noted that Chlamydomonas and Cladospora tolerated 2.0 M NaCl but grew optimally at 0.8 M NaCl. The brine shrimp, Artemia salina, isolated from the Great Salt Lake (4-5 M NaCl, Brock, 1969), required a minimum of 1.5 M NaCl for growth and grew optimally at 3.0 M NaCl (Cole and Brown, 1967).

Molds and fungi were often resistant to high salt concentrations in their environment but not many forms are reported to be strongly halophilic (Larsen, 1962). A number of strains of Sporendonema sebi (Torula epizoa, S. epizoum) grew optimally at 1.5 - 2.0 M NaCl (Frank and Hess, 1941a, b). These molds occurred frequently on salted fish, meat products, and beans (Hof, 1935). Since other salts and glucose can substitute for NaCl, these molds were really osmophilic (Vaisey, 1954).

Among the yeasts, representatives of the genus Debaromyces lived in the presence of 2.5 - 3.5 M NaCl (Hof, 1935). Salt-tolerant yeasts isolated from soy sauce (3.0 M

NaCl) and salted codfish have been reviewed by Onishi (1963), Ueno (1964), and Yoshii (1967). However, since these yeasts required a high osmotic pressure provided by various sugars, they were osmophilic and osmotolerant.

Many species of algae were characterized by their ability to require or tolerate NaCl concentrations greater than 1.0 M (Ruinen, 1938). The green algae, Dunaliella viridis, isolated from the Dead Sea, grew in media containing 1.5 to 4.0 M NaCl, although optimal growth occurred between 2.0 - 2.5 M NaCl (Elazari-Volcani, 1940, 1944; Johnson et al., 1968). D. salina grew optimally between 1.5 and 2.0 M NaCl, but tolerated, surprisingly, a saturated solution of NaCl (about 6.0 M) (Gibor, 1956). Elazari-Volcani (1943, 1944) demonstrated that a minimum of 2.5 M NaCl was required for growth by the cyanobacterium, Alphanocapsa, isolated from the Dead Sea. Batterton and Van Baalen (1971) showed that marine isolates of cyanobacteria, for example, Agmenellum quadruplicatum, tolerated 1.0 to 2.0 M NaCl compared to less than 0.5 M NaCl for non-marine isolates. Although limited, the studies on cyanobacteria suggest a distinct halotolerance.

Very little is known concerning the adaptive traits of microorganisms, other than bacteria, to NaCl. In the few cases studied, primarily algae and yeasts, an energy-requiring mechanism actively prevented high concentrations of NaCl or sugar from entering the cell, thereby preventing macromolecular alterations (Watson, 1970). When stressed with high concentrations of salts or sugars, yeasts and ciliates decreased

slightly in size, indicating possible plasmolysis, possibly caused by osmotic or ionic stresses (Brown, 1964).

Recently, an explanation has been proposed to explain the mechanism of NaCl resistance in the green alga, Dunaliella, originally isolated from the Dead Sea by Elazari-Volcani (1940). Preliminary data by Ben-Amotz and Avron (1973) indicated the osmoregulatory mechanism in Dunaliella was the result of photosynthetic production and degradation of glycerol.They noted that the intracellular concentration of glycerol varied according to the external NaCl concentration similar to the variance of intracellular K concentrations in obligate halophilic bacteria. Intracellular degradation of glycerol caused it to act as a "compatible solute", analogous to intracellular K in obligate halophilic bacteria and especially in extreme halophilic bacteria, and arabitol in osmophilic yeast (Brown and Simpson, 1972). Borowitzka and Brown (1974) found up to 40.5 % glycerol in D. viridis grown in 4.5 M NaCl. Thus, the role of glycerol, arabitol, and other solutes in extreme halophilic, nonbacterial microorganisms may be to contribute to a balanced internal osmotic pressure and maintain enzyme activity in an environment low in water activity (Ben-Amotz and Avron, 1973).

The major theme of this review was to indicate the relations between Na and microorganisms demonstrating an obligate need for this ion for growth. Two conclusions were drawn: (1) the marine bacteria studied have demonstrated highly specific, stable, and in most cases, readily detectable

requirements for Na for growth, cell envelope integrity, prevention of lysis, and substrate transport into the cells. Thus, the key to the distinction between marine and terrestrial bacteria may well be the mechanism(s) which confer on marine bacteria the selective advantage and capacity to survive and grow in the sea; (2) marine bacteria should be considered the beginning of a series of specialized microorganisms with increasing Na requirements. Extreme halophilic bacteria differed from marine and moderate halophilic bacteria. Extreme halophilism was a stable genetic character, neither acquired nor lost readily (Larsen, 1962, 1967, 1973; Kushner, 1968). However, evolutionary divergence from a common ancestor to distinct species is well known in nature (Oparin, 1962). There was a progressive dependence upon Na (and K) as illustrated by a progressively structurally simpler cell envelope, higher intracellular Na and K concentrations, and the predominance of acidic over basic proteins as environmental NaCl concentrations were increased.

Before the uniqueness of marine bacteria and extreme halophilism can be established, further questions must be answered. Studies of bacteria isolated from the fluctuating environment of an estuary might facilitate answering questions concerning the relation between Na and microorganisms indigenous to a saline environment. How stenohaline is an estuarine bacterial population? Do NaCl concentrations many fold greater than that in the estuary select for NaCl-tolerant bacteria? What are the effects of the excess NaCl

concentrations on the morphology, physiology, and biochemistry of estuarine bacteria selected for their NaCl tolerance? Investigations of these questions may contribute to an understanding of halophilism and the nature of marine bacteria.

CHAPTER III

MATERIALS AND METHODS

Experiments in this dissertation were performed in duplicate or triplicate.

A. Ecological Studies.

1. Sampling Techniques.

Samples of estuarine water were collected from the dock or the polyvinyl chloride (PVC) pipes entering the loft of the Jackson Estuarine Laboratory (JEL), Adams Point, Durham, N.H. When the dock was in the water (April-November, 1973-1975), estuarine water samples were collected manually in sterile milk-dilution bottles by complete immersion beneath the surface of the water. During the winter, water samples were collected from the PVC pipes in sterile milk dilution bottles. The pumping system for JEL was located in the basement and drew water from approximately 20 m off shore at a depth of 10 m at low tide. The seawater samples were enumerated immediately after collection. Other measurements recorded were: (1) Salinity, using either an A & O Salinometer (Goldberg Model 10423) and/or the Honeywell Electronix-15 recording chart in JEL; (2) Temperature, using either a mercury thermometer (-20 to 110 C) and/or the Leeds and Northrup Speedomax-H recording chart in JEL; (3) pH, using the Corning Model 12 pH meter.

2. Synthetic Seawater.

Two synthetic seawater formulations were used. Due to ease of preparation, Seven Seas marine synthetic seawater

(Utility Chemical Company, Paterson, N.J.) was used for the ecological studies. It had the following chemical composition (in g/liter): NaCl, 24.0; $MgSO_4 \cdot 7H_2O$, 6.0; $MgO_2 \cdot 6H_2O$, 4.5; KCl, 0.6; $CaCl_2$, 0.4; KNO_3, 0.11; $Na_2SiO_3 \cdot 9H_2O$, 0.025; KH_2PO_4, 0.02; $FeCl_3$, 0.001; $ZnCl_2$, 0.00005; $CoCl_2$, 0.000001; H_3BO_3, 0.0000004; and a one ml solution containing (in g/liter): Br, 0.065; Sr, 0.013; I, 0.00005; F, 0.00001; Tris buffer, 0.005, pH 7.6 (Biology Data Handbook, 1972). The synthetic seawater formulation had a salinity of 35.0 ± 0.5 ppt when prepared by adding 42.81 g Seven Seas marine synthetic seawater with one liter distilled water. Since water samples were taken from the Great Bay estuarine complex, the full-strength synthetic seawater was prepared to 75 % full strength to approximate the salinity of the estuary and enhance colony enumeration (ZoBell, 1941, 1946). The 75 % full-strength Seven Seas marine synthetic seawater had the following chemical composition (in g/liter): NaCl, 18.0; $MgSO_4 \cdot 7H_2O$, 4.5; $MgO_2 \cdot 6H_2O$, 3.375; KCl, 0.45; $CaCl_2$, 0.3; KNO_3, 0.0825; $Na_2SiO_3 \cdot 9H_2O$, 0.01875; KH_2PO_4, 0.015; $FeCl_3$, 0.00075; $ZnCl_2$, 0.0000375; $CoCl_2$, 0.0000007; H_3BO_3, 0.0000003; and a one ml solution containing (in g/liter): Br, 0.04875; Sr, 0.00975; I, 0.0000375; F, 0.0000075; Tris buffer, 0.00375, pH 7.6. The 75 % full-strength synthetic seawater had a salinity of 26 ± 1 ppt when prepared by adding 32.11 g Seven Seas marine synthetic seawater with one liter of distilled water. Na and Cl represented 70.8 % of the 26 ppt total salinity. The molarity of NaCl of Seven Seas formulation of salinity 26 ppt was 0.35 M.

Lyman and Fleming (L and F) synthetic seawater (1940) was used for the pure culture studies since its constituents were reagent grade chemicals which could be weighed accurately, and reproducibly. It had the following chemical composition (in g/liter): NaCl, 23.476; $MgCl_2 \cdot 6H_2O$, 10.634; Na_2SO_4, 3.917; $CaCl_2$, 1.102; KCl, 0.664; $NaHCO_3$, 0.192; KBr, 0.096; $SrCl_2 \cdot 6H_2O$, 0.040; H_3BO_3, 0.026; NaF, 0.003. The synthetic seawater had a salinity of 34.478 $\pm$ 0.100 ppt when brought to one liter with distilled water. Lyman and Fleming synthetic seawater was prepared at 75 % full strength to provide a salinity approximating that of the natural environment. The 75 % full-strength L and F synthetic seawater had the following chemical composition (in g/liter): NaCl, 17.706; $MgCl_2 \cdot 6H_2O$, 7.976; Na_2SO_4, 2.938; $CaCl_2$, 0.827; KCl, 0.498; $NaHCO_3$, 0.144; KBr, 0.072; $SrCl_2 \cdot 6H_2O$, 0.030; H_3BO_3, 0.020; NaF, 0.00225. This L and F synthetic seawater had a salinity of 26 $\pm$ 1 ppt when brought to one liter with distilled water. Na and Cl represented 85.3 % of the 26 ppt total salinity. The molarity of NaCl in the L and F synthetic seawater of salinity 26 ppt was 0.37 M. NaCl comprised 85.7 % by weight of the dissolved solids in natural seawater. The molarity of NaCl estuarine water of salinity of 26 ppt was 0.35 M (Sverdrup et al., 1942).

For the purpose of washing bacterial cells, L and F synthetic seawater (26 ppt) was used. Cells grown in medium containing less NaCl than L and F synthetic seawater were washed in the synthetic seawater made isotonic to the culture

medium with NaCl (w/v). For dry weight determinations, the bacterial cells were washed in L and F synthetic seawater made isotonic to the culture medium with 1.097 M glycerol to remove excess extracellular NaCl (MacLeod and Onofrey, 1956).

3. Media.

For the ecological studies, two seawater media were employed; modified 2216E based on that of Oppenheimer and ZoBell (1952) and modified MacLeod's Na-deficient medium based on that of MacLeod (1968).

Modified 2216E (Seven Seas)

Bacto-Peptone (Difco)	1.0 g
Bacto-Yeast Extract (Difco)	1.0 g
Seven Seas Marine Synthetic Seawater (26 ppt)	1000 ml
pH	7.4 - 7.6

When agar was employed, 16 g/liter of Bacto-Agar (Difco) was added. The medium constituents were heated until dissolved and autoclaved at 121 C for 15 min. The pH of the cooled medium was adjusted, when necessary, with either sterile 6 N NaOH or HCl to 7.4 $\pm$ 0.2 electrometrically.

Modified MacLeod's Na-Deficient Medium

Casein hydrolyzate (Nutritional Biochemical Corp.)	1.0	g
L-tryptophan (Fisher Sci. Co.)	0.03	g
Glucose (Fisher Sci. Co.)	1.0	g
p-aminobenzoic acid (Fisher Sci. Co.)	0.001	g
Niacin (Nutritional Biochemical Corp.)	0.001	g
Pantothenic acid (Sigma Chem. Co., St. Louis, Mo.)	0.001	g

(Continued)

Modified MacLeod's Na-Deficient Medium (Cont.)

Pyridoxal (K and K Lab., Plainview, N.Y.)	0.001	g
Thiamine (K and K Lab.)	0.001	g
Biotin (Sigma Chem. Co.)	0.00001	g
Cobalamine (Sigma Chem. Co.)	0.00001	g
Folic acid (Calbiochem., Los Angeles, Calif.)	0.00001	g
Special Agar (Noble)(Difco)	16.0	g
KCl (Fisher)	0.745	g
$MgSO_4 \cdot 7H_2O$ (Fisher)	6.0	g
$CaCl_2$ (Fisher)	1.11	g
Distilled water	1000	ml
Tris buffer, pH 7.5 (Fisher)	6.0	g
pH	7.4 - 7.6	

The medium constituents were heated until dissolved and autoclaved at 121 C for 15 min. The pH of the cooled medium was adjusted, when necessary, with sterile 6 N NH_4OH to 7.4 $\pm$ 0.2 electrometrically. The salt concentration of the medium as determined with a conductivity meter (Crystalab., Hartford, Conn.) was 0.0002 M.

For the pure culture studies, two seawater media were employed; modified 2216E based on that of Oppenheimer and ZoBell (1952) and glucose seawater medium.

Modified 2216E (L and F)

Bacto-Peptone (Difco)	1.0	g
Bacto-Yeast Extract (Difco)	1.0	g
L and F synthetic seawater (26 ppt)	1000	ml
pH	7.4 - 7.6	

When agar was employed, 16 g/liter of Bacto-Agar (Difco) was added. The medium constituents were solubilized by heating and autoclaved at 121 C for 15 min. The pH of the cooled medium was adjusted, when necessary, with either sterile 6 N NaOH or HCl to 7.4 $\pm$ 0.2 electrometrically.

Glucose Seawater Medium

Glucose (Fisher Sci. Co.)	1.0	g
L-glutamic acid (Nutritional Biochemical Corp.)	0.5	g
$NH_4H_2PO_4$ (Fisher)	0.05	g
K_2HPO_4 (Fisher)	0.05	g
Tris buffer, pH 7.5 (Fisher)	2.0	g
L and F synthetic seawater (26 ppt)	1000	ml
pH	7.4 - 7.6	

Adjustment of pH, when necessary, was done after autoclaving at 121 C for 15 min with either sterile 6 N NaOH or HCl to 7.4 $\pm$ 0.2 electrometrically.

4. Glassware.

Glassware was washed twice with Heikl detergent (Heinicke Corp.) in a glassware washer (Heinicke Corp.) employing a distilled water rinse of 1.5 min. For some experiments, glassware, after washing and soaking in a concentrated H_2SO_4 - HNO_3 (50 % v/v) acid bath overnight, was rinsed repeatedly with single distilled water followed by two double distilled water rinses. Glassware was air-dried and stoppered with aluminum foil.

5. Salt Addition.

NaCl addition was made on a weight to volume (w/v) basis. The total amount of NaCl in the medium or the amount supplemented to the medium was expressed as molarity. The salt(s) was added prior to the addition of nutrients and sterilization. In experiments involving stressing a growing culture with NaCl, the salt was added on a weight to volume basis to an aliquot with the amount added expressed as molarity.

No growth occurred in a NaCl-deleted glucose seawater medium in which NaCl was present as a contaminant. For this reason, the NaCl concentration in the medium and modified 2216E (L and F) medium was expressed as the salt NaCl, 0.30 M, at 26 ppt salinity.

B. Maintenance and Preparation of Standard Inoculum of Halotolerant Isolate C-1.

1. Maintenance of Culture.

Stock cultures of the halotolerant marine isolate C-1, were maintained on slants of modified 2216E (L and F) agar medium. The cultures were transferred to fresh slants every 6 - 8 weeks, incubated at 20 C for 48 h and placed in 4-6 C for storage. Prolonged preservation of the organism was accomplished by adding dimethyl sulfoxide (final concentration 10 %) to mid-logarithmic cells, and subsequently freezing the suspension in sterile screw-capped test tubes at -90 C (Manual of Microbiological Methods, 1957).

Cultures of Arthrobacter marinus (ATCC 25374), Pseudomonas cuprodurans, Pseudomonas sp. 130, and Alteromonas

marinopraesens obtained from the stock culture collection of the Department of Microbiology, University of New Hampshire, Durham, New Hampshire, were investigated for NaCl stress. All cultures were spread onto modified 2216E (L and F) agar medium and determined to be pure.

2. Inoculum.

The standard inoculum was produced from 24 h cultures grown at 20 C on slants of modified 2216E (L and F) agar medium. A loopful of the culture was inoculated into 50 ml of sterile modified 2216E (L and F) medium in a 125 ml Erlenmeyer flask, and incubated at 20 C at 200 rpm on a New Brunswick gyrotory shaking machine Model G-33, or in a Controlled Environmental Psychrotherm incubator-shaking machine, Model G-26, New Brunswick Sci. Co. At an optical density of 1.50 - 1.55, growth was harvested. Optical density measurements were performed in a Bausch and Lomb Spectronic 20 colorimeter at 420 nm). Flasks containing 100 ml of sterile test medium were inoculated with 1.0 ml of the culture washed twice in 50 ml of L and F synthetic seawater. The initial concentration of cells in the flasks was 1 to 3 x 10^7 cells/ml.

C. Photomicrography of Halotolerant Isolate C-1.

1. Phase Contrast Microscopy.

Photomicrographs were taken with a 35 mm camera (Reichert) attached to a Zeiss WL Research Microscope (Carl Zeiss). The cells were prepared as wet mounts, fixed with 10 % formalin (final concentration). Cells were observed

under 1000X phase contrast with photographs taken on Kodak Tri-X film at a quarter of a sec exposure.

2. Transmission Electron Microscopy.

a. Negative Stain and Shadow Cast.

Portions (100 ml) of modified 2216E (L and F) medium and the medium supplemented with NaCl concentrations ranging from 0.0 to 3.45 M were added to 250 ml Erlenmeyer flasks and autoclaved at 121 C for 15 min. Upon cooling, the pH was adjusted to 7.4 - 7.6 and inoculated with 1.0 ml of the standard inoculum. Flasks were incubated at 20 C and 200 rpm. Cells were harvested from the medium at the late logarithmic phase of growth by centrifugation at 10,000 x g for 15 min at 4 C in a Sorvall RC-2B Centrifuge (Sorvall Inc., Conn.). Cells were washed twice in 5 ml of synthetic seawater made isotonic to the culture medium with 2.6 M NH_4 $C_2H_3O_2$ and containing 0.1 % bovine serum albumin. One drop of cell suspension was placed on Formvar-carbon coated, 300-mesh grids (Ladd Research Ind., Vt.) for one min. Excess material was removed with Whatman #1 filter paper (W & R Balston Ltd., England). One drop of 0.5 % Na-phosphotungstate solution was placed on the grids for 5 sec and excess solution immediately removed with the filter paper (Hayat, 1970). Grids were maintained under vacuum in a desiccator at 20 C until examination using a Philips Transmission Electron Microscope (Model 200). Cells on grids were shadow cast using platinum metal at an angle of 30 degrees and 25 A thickness.

b. Thin Sections of Whole Cells and Cell Envelopes.

Cells were harvested at late logarithmic phase of growth by centrifugation at 10,000 x g for 15 min at 4 C and washed twice in 10 ml of L and F synthetic seawater made isotonic with respect to the culture medium with NaCl (w/v). The pellets were resuspended in 5 ml of the respective isotonic L and F solutions.

Cell envelopes were prepared by a modification of the method of DeVoe and Oginsky (1969 b). The washed cells were placed in stainless steel tubes, 2/3 full of acid-cleaned glass beads, and shaken in a Nossal Cell Disintegrator (Peerless Electric Co., Warren, Ohio). Shaking was in 20 sec bursts followed by immersion in an ice bath for 3 min. This procedure was repeated until examination by phase microscopy revealed 90 - 95 % broken cells. The glass beads were allowed to settle and the supernatant fluids along with two washings of the glass beads were centrifuged at 1475 x g for 20 min to remove unbroken cells. The cloudy supernates were centrifuged for 20 min at 14,500 x g to collect cell envelopes and washed twice in 5 ml of the respective isotonic synthetic seawater solutions.

Whole cells and cell envelopes were fixed and thin sectioned utilizing the following procedure. Whole cells and cell envelope suspensions were centrifuged at 15,000 x g for 15 min, resuspended in 2 ml of the respective isotonic L and F synthetic seawater containing 0.76 % redistilled glutaraldehyde (Eastman Chemical Co., Rochester, N.Y.) and allowed to

fix for 3 h at 6 C. The partially fixed cells were centrifuged at 10,000 x g for 10 min, at 4 C, washed twice with the isotonic L and F solutions and embedded in 1.0 % Ionagar No. 2 (Oxoid) prepared in L and F 26 ppt synthetic seawater. The Ionagar was washed twice in 0.1 M cacodylate-sucrose solution. The embedded material was cut into 1-2 mm^3 blocks and placed overnight in 1.0 % osmic acid in Veronal buffer pH 6.1 (Ryter and Kellenberger, 1958) prepared with 26 ppt synthetic seawater. The blocks were washed twice for 30 min periods in the Veronal buffer, then placed in 0.5 % uranyl acetate (Fisher Co., Medford, Mass.) prepared in 26 ppt synthetic seawater for 2 h at 6 C. After two more washings in the Veronal buffer, the embedded material was dehydrated through increasing concentrations of 50, 70, 90, 95, and 100 % ethyl alcohol for 30 min periods in an ice bath. The absolute alcohol was changed twice to assure complete dehydration.

The dehydrated material was placed in propylene oxide with two changes for 10 min. Each block was placed overnight at 20 C in propylene oxide:epon mixture(1:1) in an open vial. The epon mixture was prepared from equal volumes of a mixture A and B, plus 0.14 ml of 2, 4, 6-tri (dimethylaminomethyl) phenol, (DMP-30) (Ladd Research Ind., Burlington, Vt.). Mixture A was prepared with 40 g Epon resin 812 (Ladd) and 44 g dodecenylsuccinic anhydride (DDSA) (Ladd). Mixture B was composed of 50 g Epon resin 812 and 37 g nadic methyl anhydride (NMA) (Ladd). The infiltrated agar blocks were

transferred to the complete resin mixture and stored overnight at 20 C. The agar blocks were placed into empty Beem capsules (Ladd) and filled with the complete resin mixture. The capsules were placed in a 60 C oven for 48 - 72 h for polymerization. Upon returning to 20 C, the capsules were placed in a desiccator for 24 - 48 h.

The blocks were sectioned using a Porter-Blum Model MT-2 Microtome (Sorvall, Inc., Norfalk, Conn.) and post-stained with lead citrate and uranyl acetate (Hayat, 1970).

3. Scanning Electron Microscopy.

Cells were washed and fixed in the 0.76 % glutaraldehyde-seawater solution. The fixed cells were washed twice in the isotonic synthetic seawater solutions, placed on clean 22 mm^2 glass coverslips, air dried, and dehydrated through increasing concentrations of 50, 70, 90, 95, and 100 % ethyl alcohol for 15 min periods in an ice bath. The coverslips were placed in amyl acetate for 15 min and critical point dried (Broers et al., 1975). The dried specimens were plated (Pd-Au 40:60) and examined using an AMR-1000A Scanning Electron Microscope.

D. Identification of Halotolerant Isolate C-1.

Cultural characteristics of the halotolerant bacterium were determined using the criteria appearing in Colwell and Wiebe (1970), Meynell and Meynell (1970), Skerman (1967), and the Manual of Microbiological Methods (1957). Media were prepared with L and F 26 ppt synthetic seawater with supplemental NaCl concentrations ranging from 0.0 to 3.45 M

and incubated at 20 C. Cultural characteristics were performed on C-1 at supplemental NaCl concentrations to confirm the identity of the bacterium and the purity of the culture. The following stains were prepared according to Meynell and Meynell (1970).

Acid-fast stain: by Carbol Fuchsin.

Capsule stain: by Indian ink.

Fat stain: by Sudan Black.

Flagellum stain: by Leifson Flagella stain.

Gram stain: by Hucker modification.

Spore stain: by Malachite green.

Media used for growth characteristics were:

Extract Agar (BBL), Extract Broth (BBL), modified 2216E broth, and modified 2216E agar medium were prepared with 26 ppt L and F synthetic seawater. Bacto-Marine Agar 2216 (Difco) was prepared in distilled water.

The following cultural characteristics were determined using the criteria set forth in Skerman (1967).

Indole production was determined by cultivation in Trypticase Soy Broth (BBL). Indole was tested for by Kovac's reagent (1.0 % para-dimethylaminobenzaldehyde, 82 % butyl alcohol, and 17 % concentrated HCl).

Catalase was determined by the addition of one drop of 3 % H_2O_2 to colony growth on modified 2216E (L and F) agar medium

Kovac oxidase activity was determined by streaking a loopful of growth from modified 2216E (L and F) agar medium

onto Whatman #1 filter paper (W & R Balston, Ltd., England) saturated with N, N, N, N-tetramethylparaphenyl-diamine HCl.

The following cultural characteristics were determined using the criteria set forth in the Manual of Microbiological Methods (1957).

Nicotine utilization was determined by cultivation on nicotine agar prepared according to Sguoros (1955); nicotine (Eastman Co., Rochester, N.Y.), 0.4 %; KH_2PO_4, 0.2 %; KCl, 0.5 %; $MgSO_4$, 0.0025 %; $FeSO_4$, 0.0025 %; Bacto-Yeast Extract (Difco), 0.1 %; Bacto-Agar (Difco), 1.5 %.

Gelatin hydrolysis was determined by two techniques: (1) Cultivation on Extract Agar (BBL) containing 0.4 % Bacto-Gelatin (Difco). Gelatin hydrolysis was tested for by flooding the plates containing colonies with 15 % acid $HgCl_2$ solution. Transparent zones surrounding colonies indicated gelatin hydrolysis. (2) Cultivation in stab cultures prepared with Extract Broth containing 12 % Bacto-Gelatin (Difco).

Starch hydrolysis was determined on plates of Starch Agar (Difco) by flooding plates with iodine solution every two days for 12 days.

Nitrate reduction was determined by cultivation in Trypticase Soy Broth (BBL) containing 0.1 % KNO_3. Nitrate was tested for by Griess-Ilosvay reagent.

H_2S production was determined by cultivation on Lead Acetate Agar slants (Difco) and Extract Agar (BBL), containing

0.1 % cysteine and 0.02 % $FeSO_4 \cdot 7H_2O$. Blackening of the Lead Acetate Agar slants indicated the production of H_2S.

Citrate utilization was determined by cultivation on Simmons Citrate Medium (BBL) and in Koser's Citrate Broth (Difco).

Urease production was determined by cultivation in Bacto-Urea Broth (Difco). Hydrolysis of urea was tested for by a pH rise and subsequent color change.

Litmus milk reaction was determined by cultivation in Bacto-Litmus Milk (Difco) and brom cresol purple milk.

Acetylmethylcarbinol production was determined by cultivation in MR-VP Medium (BBL) and tested for by Baritts Method.

Ammonium and nitrate utilization as sole source of nitrogen was determined by cultivation in media composed of glucose, 1.0 %; K_2HPO_4, 0.005 %; and either NH_4Cl, or KNO_3, 0.1 %.

Creatinine and creatine utilization as sole source of nitrogen--and/or carbon was determined by cultivation in media prepared according to Dubos and Miller (1937); K_2HPO_4, 0.005 %; and either creatinine, or creatine, 0.2 %. For carbon source utilization, $(NH_4)_2SO_4$, 0.05 % was added to the medium.

Carbohydrates and carbon compounds as sole source of carbon was determined by cultivation in $NH_4H_2PO_4$, 0.005 %; K_2HPO_4, 0.005 %; and either glucose, glycerol, ribose, Na-acetate, Na-lactate, or Na-citrate, 0.5 %.

Carbohydrate utilization was determined by cultivation in Bacto-Phenol Red Broth (Difco) with 0.5 % carbohydrate as well as in a synthetic base medium composed of NH_4NO_3, 0.1 %; K_2HPO_4, 0.002 %; phenol red, 0.002 %; asparagine, 0.002 %; and carbohydrate, 1.0 %. Inverted Durham tubes were placed in tubes containing both media.

Cellulose utilization was determined by cultivation on Whatman # 1 filter paper (W & R Balston, Ltd., England) soaked in a medium composed of NH_4NO_3, 0.1 %; K_2HPO_4, 0.02 %; and asparagine, 0.002 %.

Growth in a nitrogen free medium was determined by cultivation on Ashby's nitrogen-free medium prepared with mannitol, 1.5 %; $MgSO_4 \cdot 6H_2O$, 0.02 %; K_2HPO_4, 0.02 %; NaCl, 0.01 %; $CaSO_4 \cdot 2H_2O$, 0.01 %; $CaCO_3$, 0.5 %; and Special Agar (Noble) (Difco), 1.5 %.

Thiotone utilization was determined by cultivation in Thiotone broth prepared according to Blankenship and Doetsch (1961); Thiotone (BBL), 0.1 %; glucose, 0.25 %; and K_2HPO_4, 0.25 %.

Growth under anaerobic conditions was determined by cultivation in modified 2216E (L and F) medium and on modified 2216E (L and F) agar medium in a Brewer anaerobic jar (BBL) made anaerobic with a disposable CO_2 gas-pak (BBL).

The following cultural characteristics were determined using the criteria set forth in Colwell and Wiebe (1970).

The growth response to temperature (0-45 C) and pH (4.0 - 9.0) was determined by cultivation in modified 2216E (L and F) medium and on modified 2216E (L and F) agar medium. The pH was adjusted after autoclaving at 121 C for 15 min with either sterile 6 N HCl or NaOH.

Antibiotic reaction was determined by cultivation on modified 2216E (L and F) agar medium containing sterile Bacto-Sensitivity Antibiotic Disks (Difco). Vibriostat 0/129 (pteridine) (Shewan et al., 1954) was used to differentiate vibrio-type from pseudomonad-type organisms. Pseudomonads are insensitive to 0/129.

Fluorescein production was determined by cultivation on either Pseudomonad Agar-F (Difco) or Pseudomonad Agar-P (Difco) (King et al., 1954) under ultra-violet light (#G8TS, General Electric Co., and F8TS-BLB, Westinghouse Co.).

Nitrogenous metabolism was determined by cultivation on arginine dihydrolase medium 2A prepared according to Thornley (1960); Bacto-Peptone, 0.1 %; arginine HCl, 1.0 %; K_2HPO_4, 0.03 %; phenol red, 0.001 % and Bacto-Agar (Difco), 0.3 %. Lysine Decarboxylase Medium (Difco) was also used to determine nitrogenous metabolism.

Tween 80 utilization was determined by cultivation on a medium composed of Bacto-Yeast Extract (Difco), 0.1 %; Bacto-Peptone (Difco), 0.1 %; Tween 80 (Fisher Co.), 1.0 %; and Bacto-Agar (Difco), 1.5 %.

Phosphatase production was determined by cultivation on a medium prepared according to Baird-Parker (1963); of Casein hydrolyzate, (Nutritional Biochemical Corp.), 0.2 %; Bacto-Beef Extract (Difco); 0.2 %; phenolphthalein diphosphate (Na salt), 0.0001 %; Bacto-Agar (Difco), 1.5 %. Phosphatase production was tested for by the appearance of deep pink colonies upon exposure to one drop of 6 N NH_4OH.

Accumulation and hydrolysis of poly-β-hydroxybutyrate was determined by cultivation in a chemically defined medium prepared according to Stanier et al., (1966); $(NH_4)_2SO_4$, 0.02%; KH_2PO_4, 0.02 %; K_2HPO_4, 0.02 %; and either DL-β-hydroxybutyrate, 0.5 %; or DL-β-hydroxybutyrate, 0.5 %; and Na-acetate, 0.5 %. Accumulation of poly-β-hydroxybutyrate was tested for with Sudan Black.

Molar percent guanine plus cytosine (GC) of DNA was determined by cultivation in modified 2216E (L and F) medium and the medium supplemented with 1.70, 2.60, and 3.45 M NaCl to late logarithmic growth and DNA extracted by the method of Marmur (1961), modified to include phenol and predigested Pronase (Calbiochem. Co.) to insure more complete protein removal (Colwell and Wiebe, 1970). The GC molar % was determined by the methods of thermal melting (Marmur and Doty, 1962) and buoyant density (Schildkraut et al., 1962). Appreciation is extended to Dr. Dean at

Brandeis University for the determination of guanine plus cytosine molar % using the buoyant density technique.

DNA-DNA hybridization studies were determined by cultivation in modified 2216E (L and F) medium and this medium supplemented with 2.6 M NaCl to late logarithmic growth and extracted by the method of Marmur (1961) modified to include phenol and Pronase (Colwell and Wiebe, 1970). Labelled DNA was prepared by the method of Citarella and Colwell (1970) using 3 μCi/ml carrier-free $H_3{}^{32}PO_4$ (Tracerlab Co.) added to the cultures at an optical density of 0.10. Unlabelled DNA was extracted also from simultaneously inoculated medium. The DNA-DNA membrane filter hybridization was performed by the method described in Miller (1972).

E. Investigations of Isolate C-1 in the Presence of NaCl.

1. Respiration Experiments.

Oxygen utilization was measured using standard Gilson methods outlined in Umbreit et al. (1964). Respiration was measured with the use of the GR-20 Gilson Differential respirometer (Gilson Medical Electronic, Milwaukee, Wisc.) employing a reciprocal shaking speed of 140 rpm at 20 C.

Cell suspensions were prepared by adding 100 ml modified 2216E (L and F) medium and supplemented with 1.70 and 2.60 M NaCl to 250 ml Erlenmeyer flasks. Flasks were autoclaved at 121 C for 15 min, pH adjusted to 7.4 - 7.6, and inoculated with 1.0 ml of the standard inoculum. After growth to the

late logarithmic phase at 20 C and 200 rpm, cells were harvested by centrifugation at 10,000 x g for 15 min at 4 C. The cell pellets were washed twice with 15 ml of synthetic seawater made isotonic to the growth medium with NaCl (w/v), and the final pellets suspended in 15 ml of L and F synthetic seawater. The cell suspension from modified 2216E medium was divided into a 5 ml and a 10 ml portion: (1) The 5 ml portion was centrifuged once more at 10,000 x g for 15 min at 4 C and resuspended in 5 ml of synthetic seawater made isotonic to the growth medium with 1.097 M glycerol. This portion was used to study respiratory activity in the presence of total NaCl concentrations of 0.01, 0.1, 0.2, and 0.25 M. (2) The 10 ml portion was used to study respiratory activity in the presence of total NaCl concentrations ranging from 0.3 to 5.45 M.

The following components were added to each respiration flask prior to equilibration at 20 C for 15 min: 1.0 ml of cell suspension (initial concentration, 3 - 7 x 10^{10} cells/ml), 1.0 ml of L and F synthetic seawater made isotonic to 26 ppt seawater with 1.097 M glycerol, 1.0 ml of 3 x concentration of NaCl-deleted modified 2216E medium (added to the side-arm of the flask), and 0.2 ml of 2 M KOH (added to the center-well of the flask). Pre-sterilized NaCl was added to each flask (w/v) to give final concentrations ranging from 0.01 to 5.45 M after 15 min equilibration. The experimental timing was started with the addition of modified 2216E medium to the bacterial suspension. Endogenous flasks contained 0.2

ml of 2 M KOH, 2.0 ml of L and F synthetic seawater, and 1.0 ml of cell suspension in L and F seawater. Thermobarometers contained 3.0 ml of L and F seawater and 0.2 ml of 2 M KOH.

Respiration readings were taken every 10 min for 3 h with dry weights determined. Dry weight determinations were obtained from 0.5 ml aliquots of the cell suspension taken from the respiration flasks at the start and finish of the experiment. Cells were washed twice in L and F synthetic seawater made isotonic to the flask medium with 1.097 M glycerol to remove excess extracellular Na. The washed cell suspensions (1.0 ml) were placed in tared aluminum weighing pans maintained at 110 C for 24 - 48 h until a constant dry weight was obtained.

2. Triphenyl Tetrazolium Chloride Overlay Technique for Determining Respiratory Sufficiency or Deficiency of C-1.

The halotolerant isolate was grown on modified 2216E (L and F) agar medium with total NaCl concentrations ranging from 0.01 to 3.75 M for 10 days at 20 C. The resulting colonies were overlayed with 0.1 % triphenyl tetrazolium chloride (TTC) in either 1.5 % agar or 10 % gelatin and reincubated an additional one h at 20 C (Lindegren et al., 1958).

The agar and gelatin solutions were prepared in L and F synthetic seawater, autoclaved at 121 C for 15 min, and dispensed in 14 ml amounts into sterile test tubes. TTC was prepared separately by adding 0.8 g of TTC to 100 ml of L and F synthetic seawater and filter sterilized through 0.45 µm filters (Millipore Filter Apparatus, Millipore Corp., Bedford, Mass.). The complete overlay was prepared by adding 2.0 ml

of the 0.8 % TTC solution to either 14 ml of 50 C molten agar or 37 C gelatin.

3. Thunberg Studies.

Portions (100 ml) of modified 2216E (L and F) medium with total NaCl concentrations ranging from 0.01 to 3.75 M were added to 250 ml Erlenmeyer flasks. The flasks were autoclaved at 121 C for 15 min, pH adjusted to 7.4 - 7.6, and inoculated with 1.0 ml of the standard inoculum. Flasks were incubated at 20 C and 200 rpm. Upon attaining the late logarithmic phase of growth, the cells were harvested by centrifugation at 10,000 x g for 15 min at 4 C. The cell pellets were washed 3 times with 50 ml of synthetic seawater made isotonic to the growth medium with NaCl (w/v). The final pellets were resuspended in 5 ml of isotonic L and F synthetic seawater solutions (2 - 3 x 10^{11} cells/ml).

TTC, methylene blue (MB), and dichloroindophenol (DCIP) employed as oxidation-reduction dyes to measure rates of electron transport in the halotolerant isolate were prepared by adding 0.003 g of the dye to 100 ml of L and F synthetic seawater made isotonic to the growth medium with NaCl (w/v).

The following components were added to each Thunberg tube prior to equilibration at 20 C for 20 min: 1.0 ml of dye (initial concentration 0.0005 %) 1.0 ml of 0.1 M PO_4 buffer, both prepared in the respective isotonic L and F synthetic seawater solutions, 2.0 ml of 3 x concentration of modified 2216E medium containing the same NaCl concentration as the growth medium (0.01 to 3.75 M), and 2.0 ml of the washed cell

suspension added to the side-arm of the tube. The tubes were evacuated by use of a vacuum pump for 5 min, sealed, and the experiment started by mixing the cell suspension with the other components (Umbreit et al., 1964). Optical densities were performed on the tubes incubated at 20 C, using a Bausch and Lomb Spectronic 20 colorimeter against blanks containing the respective dyes and all components including a formalized cell suspension. Reduction of TTC was measured at 590 nm, MB and DCIP reduction were measured at 660 nm.

4. Qualitative Analysis of Cytochromes on Isolate C-1.

Five hundred ml of modified 2216E (L and F) medium and this medium supplemented with 2.6 M NaCl were added to one-liter Erlenmeyer flasks. The flasks were autoclaved at 121 C for 15 min, pH adjusted to 7.4 - 7.6, and inoculated with 5 ml of the standard inoculum. Flasks were incubated at 20 C at 200 rpm.

Upon attaining the late logarithmic phase of growth, the cells were harvested by centrifugation at 10,000 x g for 15 min at 4 C. The cell pellets were washed 3 times with 50 ml of L and F synthetic seawater made isotonic to the growth medium with NaCl (w/v). The final pellets were resuspended in 5 ml of isotonic L and F synthetic seawater solutions (1 - 2 x 10^{12} cells/ml).

The final cell suspensions were disrupted by sonication employing a 100 watt M.S.E. sonicator (Measurement Scientific Electronics, London) with a sonic amplitude of 4 μ peak to peak. Six 1.0 min treatments were employed with a 1.0 min

cooling time between treatments. Upon completion of the sonication process, wet mounts of the suspensions were prepared and examined under the phase microscopy at 1,000 x magnification. The disruption process was considered complete if three or less intact cells per phase field were observed. If the disruptive process was incomplete, then two additional sonication treatments were employed.

Following disruption the suspensions were centrifuged at 3,500 x g for 30 min to remove the residual intact cells. Following this centrifugation, the supernates were removed and saved for spectrophotometric analyses of the cytochromes.

a. Difference Spectrum.

Qualitative measurements of the cytochromes were performed on the whole cell extracts employing the difference spectrum technique of Chance (1954). In this procedure, the test cuvette was oxidized initially with 0.01 M K_3 $Fe(CN)_6$, then reduced by the addition of a few crystals of sodium dithionite ($Na_2S_2O_4$).

b. Absorption Spectrum.

Qualitative measurements of the cytochromes were performed on the whole cell extracts employing the absorption spectrum technique of Umbreit et al. (1964). In this procedure, the contents of the test cuvette were reduced by $Na_2S_2O_4$ and compared to a blank containing all components except the cell extract.

All spectral analyses were performed using a Cary Model 15 Spectrophotometer employing the one-tenth scale. A scan of 400 to 650 nm was employed for all spectral analyses.

5. Intracellular Determination of Na and K on Isolate C-1.

Portions (1,000 ml) of modified 2216E L and F medium and the medium supplemented with 1.30 and 2.60 M NaCl were added to acid-cleaned 2-liter Erlenmeyer flasks and autoclaved at 121 C for 15 min. Upon cooling, the pH of the medium was adjusted to 7.4 - 7.6 and inoculated with 10 ml of the standard inoculum. At the early, mid-, and late logarithmic phases as well as the stationary phase of growth after incubation at 20 C and 200 rpm, 200 ml aliquots were removed and harvested by centrifugation at 10,000 x g for 15 min at 4 C. Cells were washed twice and the final pellet suspended in 30 ml of L and F synthetic seawater made isotonic to the growth medium with NaCl (w/v), such that a 10 ml sample yielded a pellet of 250 - 450 mg cell wet weight or almost 80 - 150 mg cell dry weight (Takacs et al., 1964).

Cell suspensions containing 25 - 45 mg wet weight per ml were prepared in 350 ml (60 x 140 mm) wide-mouth polycarbonate centrifuge bottles and shaken at 250 rpm and 20 C to insure vigorous aeration during the 30 min incubation period. Sets of three 10 ml samples were removed for intracellular Na and K analysis.

Each 10 ml thick suspension sample for ionic analyses was pipetted into a 50 ml (29 x 104 mm) polycarbonate centrifuge tube, in which the extractions were to be carried

out. The tubes were centrifuged at 15,000 x g for 15 min at 4 C. The resulting supernates were discarded and the inside walls of the tubes rinsed carefully with double distilled (deionized) water from a squeeze bottle to remove residual ions from the supernate. The inside walls of the tubes were wiped with Kimwipes and allowed to sit until the pellet wet weights were determined.

The pellet was resuspended in 2.5 ml of double distilled water by extensive mechanical "vortex" agitation until no large pellet clumps remained. Finally, 2.5 ml of 0.62 M trichloroacetic acid (TCA) was added to each resuspended pellet (the final extraction solution contained 0.31 M TCA). The polycarbonate tubes were shaken for 5 min at 200 rpm and 20 C and immersed in a 95 - 99 C water bath for 5 min. The tubes containing the extract were then centrifuged at 15,000 x g for 15 min at 4 C and the supernates decanted into plastic or polycarbonate containers for later dilution and analysis. The flocculant precipitated material did not contain any significant ionic content, indicating that the extraction procedure was essentially 100 % efficient in solubilizing cellular ions. Care was taken during the procedures that all solutions, including the pellets and extracts, did not contact materials which might add contaminating ions. To this end, all glassware was acid-cleaned in a 1:1 (50/50, v/v) concentrated sulfuric-nitric acid bath for 18 - 24 h.

Extracts were diluted appropriately with double distilled water using Oxford Sampler micropipettes (volume

calibrations checked before use) with plastic tips and 1 ml Fisher plastic mini-beakers and caps. A Varian-Techtron AA-5 Atomic Absorption Spectrophotometer was used for flame emission analyses of Na and K. The gas was acetylene/air, the wavelengths were 5893 A and 7665 A for concentration ranges of 0-1 mg/ml, for Na and K, respectively. Ionic content of diluted extracts was determined from the mean of triplicate determinations. To obtain a matrix similar in composition to the sample, standards for Na and K were made from stock solutions (Na, K, Atomic Absorption Standards, 1000 ppm, Fisher Sci. Co., Medford, Mass.) diluted with 0.62 M TCA (final TCA concentration was 0.31 M).

The 10 ml thick suspension samples for extracellular fluid volume determinations were pipetted into 15 ml calibrated, glass centrifuge tubes which contained 1 ml of a solution of a radioactive solute which could not penetrate into the cells. New England Nuclear Corp. inulin carboxyl-14 C (NEC 164P) was used as the non-penetrating solute and made in the same solution at the same NaCl concentration as the thick suspension (3 %, 1 μCi/ml). The amount of labelled solute (with extra nonlabelled carrier solute added when the concentrations of labelled substance were low) was adjusted to give adequate counts (1000 - 5000 DPM) from the S_2 supernatant aliquot (see below). The tubes containing the suspension and labelled substance were mixed by vortexing, then centrifuged at 15,000 x g for 15 min at 4 C. The resulting supernatant, S_1, was decanted and saved. The height of the resulting packed cells

was measured and the volumes determined. The inside tube walls were rinsed with double distilled water using a squeeze bottle to remove adhering radioactive substance, and the walls wiped with Kim-wipes. Five ml of the same solution except without the labelled substance was added to each pellet and the pellets resuspended. The suspensions were centrifuged at 15,000 x g for 15 min at 4 C and supernatant, S_2, decanted and retained. Aliquots (0.2 ml) of S_1 and S_2 were pipetted into scintillation vials to which 10 ml New England Nuclear Aquasol cocktail had been added. Counting was done in a Packard Tri-carb Liquid Scintillation Spectrometer, Model 3330. Aliquots (1 ml) of supernate 1 were diluted with double distilled water and Na and K determined by flame photometry.

The extracellular fluid volume, i.e., the volume of solution trapped in the first pellet, was determined using the following equation (Takacs et al., 1964):

$$XS_1 = (V-v+X)S_2$$

Where X= volume of extracellular fluid associated with the packed cells (ml)

S_1= inulin carboxyl-^{14}C concentration (determined by DPM) in supernate 1

S_2= inulin carboxyl-^{14}C concentration (determined by DPM) in supernate 2

V= total volume of the suspension (ml)

v= total volume of the packed cells (ml)

The intracellular fluid volume was estimated by subtracting from the volume of the packed cells, the sum of the

extracellular fluid volume and the volume occupied by the dry weight of the cells, assuming the latter to have a density of 1.0.

The third, and final, 10 ml thick suspension samples were used for wet and dry weight determinations. The samples were centrifuged at 15,000 x g for 15 min at 4 C in tared 50 ml polycarbonate centrifuge tubes. The supernates were discarded, the inside tube walls rinsed with double distilled water, and wiped with Kim-wipes. A jet of gently flowing dry air was directed inside the tubes until the walls were dry (1 - 2 min). The tubes were weighed and the wet weights of the pellets determined. After 24 h at 100 C, the tubes were reweighed and the dry weights of the pellets determined.

Intracellular Na and K concentrations were calculated using the following equation (Takacs et al., 1964):

$$FG = 1{,}000H - IJ$$

Where F= intracellular Na or K concentration (mM)

G= intracellular fluid volume (ml)

H= total Na or K associated with the packed cells (mM)

I= extracellular fluid volume (ml)

J= concentration of Na or K in supernate 1 (mM)

Total Na in the packed cell was also determined by a ^{22}Na ratio procedure (Takacs et al., 1964). The experimental protocol utilizing ^{22}Na will be discussed in the following section. Total Na was calculated by solving for E in the following equation (Takacs et al., 1964):

$$ABC - DE$$

Where A= ^{22}Na activity per ml of packed cells

B= ^{22}Na concentration in supernate 1 as determined by flame photometry

C= total volume of the packed cells (ml)

D= ^{22}Na activity per ml of supernate 1

E= total Na associated with the packed cells (mM)

Total K associated with the packed cells was determined by direct flame photometry only. Na and K concentrations in the uninoculated medium were determined with 5 ml aliquots analyzed by direct flame photometry.

6. Determination of Na Associated With Cells of Isolate C-1 Using ^{22}Na.

The use of the ^{22}Na ratio procedure as well as the determination of ^{22}Na associated with C-1 cells was a result of the following experiment, which was performed at the same time as the preceding experiment.

Portions (500 ml) of modified 2216E (L and F) medium and this medium supplemented with 1.3 and 2.6 M NaCl were added to acid-cleaned one-liter Erlenmeyer flasks, autoclaved at 121 C for 15 min, and the pH adjusted to 7.4 - 7.6. Prior to inoculation with 5 ml of the same standard inoculum as used in the preceding experiment, medium containing 0.0, 1.3, and 2.6 M NaCl was seeded with ^{22}Na (as $^{22}NaCl$) to initial concentrations of 0.006 μCi/ml (2.22×10^{-2} nM), 0.03 μCi/ml (11.1×10^{-2} nM) and 0.06 μCi/ml (22.2×10^{-2} nM), respectively. The increased concentrations of ^{22}Na were added to maintain a

relatively constant ratio between labelled and unlabelled Na in the medium. Flasks were incubated at 20 C and shaken at 200 rpm. At 1 and 2 h after inoculation, 10 ml aliquots were pipetted into 5 ml calibrated polycarbonate glass centrifuge tubes, washed twice in 5 ml of L and F synthetic seawater made isotonic to the growth medium with NaCl (w/v) by centrifugation at 10,000 x g for 15 min at 4 C. The final pellet was resuspended in 3 ml of the respective isotonic L and F seawater. One ml was placed in tared aluminum weighing pans for dry weight determinations. Pans were maintained at 110 C for 24 h. Two ml were saved for further analysis of ^{22}Na activity. In addition, 100 ml aliquots removed at early, mid-, and late logarithmic phases and at the stationary phase of growth were pipetted into 40 ml calibrated glass centrifuge tubes, washed twice and resuspended in 60 ml of the respective isotonic L and F solution by centrifugation at 10,000 x g for 15 min at 4 C. Each 60 ml suspension was subdivided into four 10 ml aliquots and a 20 ml aliquot. Each 10 ml aliquot was treated and analyzed for cell-associated Na as described in the previous section: aliquot 1 treated with 0.62 M TCA to determine ^{22}Na activity per ml of packed cells; aliquot 2 determined wet and dry weight; aliquot 3 treated with 3 % inulin carboxyl-^{14}C (1 μCi/ml) to determine ^{22}Na concentration in S_1 by flame photometry, and aliquot 4 treated with 3 % inulin carboxyl-^{14}C (1 μCi/ml) to determine ^{22}Na activity in S_1. The 20 ml aliquot was centrifuged at 15,000 x g for 15 min at 4 C and resuspended in 5 ml of the respective isotonic

L and F seawater. The 5 ml samples were shaken in a Nossal Disintegrator to extract cell envelopes as described in C-2b and were saved to determine ^{22}Na activity in the cell envelopes. Triplicate 0.5 ml samples from aliquots saved for analysis of ^{22}Na activity were placed in plastic gamma counting tubes and assayed using a Packard Auto Gamma Spectrometer accessory to the Tri-Carb Liquid Scintillation Spectrometer, model 3330, previously calibrated with Cesium-137.

The precise amount of radioactivity available in the medium was assayed in the same manner as the cell extracts, with 0.5 ml aliquots removed prior to inoculation.

F. Biochemical Responses of C-1 to NaCl.

1. Determination of DNA: RNA: Protein: Carbohydrate: Phospholipid Ratios on Isolate C-1.

Duplicate 100 ml portions of modified 2216E (L and F) medium with supplemental NaCl concentrations ranging from 0.0 to 3.45 M were added to 250 ml Erlenmeyer flasks and autoclaved at 121 C for 15 min. Upon cooling, the pH was adjusted to 7.4 - 7.6 and the medium inoculated with 1.0 ml of the standard inoculum. The inoculated medium was incubated at 20 C and 200 rpm with 25 ml aliquots removed at the early, mid-, and late logarithmic phases and at the stationary phase of growth. The cells were centrifuged twice at 10,000 x g for 15 min at 4 C and resuspended in 25 ml of L and F synthetic seawater made isotonic to the growth medium with NaCl (w/v). Prior to extraction, duplicate 0.5 ml aliquots of the cell

suspensions were mixed with 0.5 ml of 5 % formalin for direct cell counts.

The 25 ml aliquots were divided into 20 ml and 5 ml fractions and transferred to polycarbonate centrifuge tubes. Tubes were centrifuged at 10,000 x g for 15 min at 4 C with the supernates discarded. The pellets from the 5 ml fractions were resuspended in 5 ml of 1N H_2SO_4, stoppered and stored at 4 C for total carbohydrate determination. The pellets from the 20 ml fractions were resuspended in 5 ml cold 0.62 M TCA, mixed, and allowed to stand at 4 C for 10 min. The suspensions were centrifuged at 5,000 x g for 15 min at 4 C, discarding the supernates and redissolving the pellets in 5 ml cold 95 % ethyl alcohol. After 10 min, the suspensions were centrifuged again at 5,000 x g for 15 min at 4 C. The supernates were saved for phospholipid analysis. The pellets were resuspended in 2.5 ml cold 0.62 M TCA and 2.5 ml distilled water. The suspensions were heated at 90 - 100 C for 15 min with occasional stirring. Upon cooling, the suspensions were centrifuged at 10,000 x g for 20 min at 4 C with the supernates saved for DNA and RNA analysis. The pellets were resuspended in 5 ml of 0.1 N NaOH and saved for total protein determination.

DNA was determined by the method of Burton (1956), using calf thymus DNA (Calbiochem) as a standard.

RNA was determined by the method of MejBaum (1939) employing the orcinol test, and RNA (Sigma) as a standard.

Protein was determined according to the Lowry method (Lowry et al., 1951) using bovine serum albumin fraction V (Sigma) as a standard.

Carbohydrate was determined by the anthrone test of Seifer et al. (1950) with glucose as a standard.

Phospholipid was determined by the micro-Kjeldahl method of Fiske and Subbarow (1925).

2. Amino Acid and Phospholipid Determination on Cell Envelopes of Isolate C-1.

Portions (200 ml) of modified 2216E (L and F) medium and this medium supplemented with 1.70 and 3.45 M NaCl were added to 500 ml Erlenmeyer flasks and the flasks autoclaved at 121 C for 15 min. Upon cooling, the pH of the medium was adjusted to 7.4 - 7.6 and the medium inoculated with 2.0 ml of the standard inoculum. Cell envelopes were prepared from cells grown to the stationary phase of growth at 20 C and 200 rpm as described in C-2b.

Weighed amounts of crude envelope preparations were transferred to 100 ml dilution bottles containing 60 ml of a buffered enzyme solution having the following composition: 0.25 M Tris buffer, pH 7.8, (Fisher); 0.05 % trypsin (Worthington); 0.5 % RNase (Worthington); and 0.005 % DNase (Calbiochem) in 1000 ml of L and F synthetic seawater made isotonic to the growth medium with NaCl (w/v). Small clumps of envelope material were broken apart by rapid expulsion of the mixture through a 10 ml syringe and a 20 gauge needle. The solutions were allowed to incubate at ambient temperature

(20 C) for 2 - 8 h and centrifuged at 14500 x g for 20 min at 4 C. The pellets were resuspended in 5 ml of the respective isotonic L and F seawater and stored at 4 C.

a. Amino Acid Analysis.

Hydrolysis of cell envelope preparations was by the methods of Hill (1965) and Laemmli (1970). Ten 100 mg portions of cell envelope were added to an acid-cleaned test tube heated previously and drawn down to a small opening. Five ml of 6 N redistilled HCl (for 50 mg or less of cell envelope) or 10 ml of 6 N redistilled HCl (for 50 mg or more of cell envelope) was added to the tube. One drop of hydrazine-HCl was added to each tube. All tubes were heat-sealed under vacuum. Sealed tubes were incubated at 110 C for 24 - 36 h.

The hydrolyzates were filtered over sintered-glass to remove insoluble materials. The filters were washed twice with 10 ml of distilled water, the filtrate plus washes placed in an acid-cleaned 25 ml Erlenmeyer flask, and dried in air over 6 N NaOH.

For amino acid analyses, 4 ml of 0.2 M sodium citrate buffer, pH 2.2, was added to each tube and the amino acid composition determined on 0.5 ml samples in a Beckman Model 120 C Amino Acid Analyzer.

b. Phospholipid Analysis.

Phospholipid analysis of 5 - 10 mg of cell envelope preparations was by the method of Folch et al. (1957). Samples were extracted twice in a 20-fold volume of

chloroform-methanol (2:1) and filtered. Filtrates were dried (in vacuo), extracted twice with 20 ml of water and redissolved in 5 ml of chloroform-methanol (2:1). The lipids were precipitated with a 10-fold volume of acetone, collected by centrifugation at 7500 x g for 15 min at 4 C, redissolved in 7.5 ml of chloroform-methanol (2:1), and stored in sealed ampoules under N_2 at -20 C. The lipid extracts were chromatographed by spotting 50 µg (approximately 1 mg/ml lipid) on Eastman silica gel thin layer sheets (Dixillation Products Industries, Rochester, N.Y.). Spots were revealed with iodine vapors, molybdate blue, and ninhydrin-lutidine-n-butyl alcohol spray reagent (Abramson and Belcher, 1964). Individual spots were eluted with a minimum volume of chloroform-methanol (2:1). Eluates were spotted on silica gel sheets and developed in chloroform-methanol-acetic acid-water (65:25:12:4, v/v). The phospholipids used as standards were phosphatidylethanolamine, phosphatidylserine, diphosphatidylglycerol, phosphatidylcholine and phophatidic acid (Fisher Sci. Co., Medford, Mass.).

3. Extraction of the Enzyme Aspartate Transcarbamylase from Isolate C-1.

Portions (500 ml) of modified 2216E (L and F) medium and this medium supplemented with 2.6 M NaCl were prepared, placed in one-liter Erlenmeyer flasks, and autoclaved at 121 C for 15 min. Upon cooling, the pH was adjusted to 7.4 - 7.6 and 5 ml of the standard inoculum added to the medium. Flasks were incubated at 20 C and 200 rpm. Cells were harvested at

the stationary phase of growth by centrifugation at 10,000 x g for 15 min at 4 C and washed twice in 15 ml of the respective isotonic L and F seawater to give approximately 3 mg protein per ml as determined by Lowry et al. (1951). Cells in the suspension were disrupted by sonication employing a 100 watt M.S.E. Sonicator (Measurement Scientific Electronics, London) with a sonic amplitude of 6 μ peak to peak. Eight 1.0 min treatments were employed with 1.0 min cooling time between treatments to yield 90 - 95 % cellular disruption as determined by phase microscopy. Following disruption, cells were centrifuged at 3500 x g for 30 min at 4 C to remove residual intact cells. Following this centrifugation, the supernate was removed and saved for analysis.

The supernate constituted the crude extract. Enzyme extraction and precipitation was performed using the method of Kaplan et al. (1967). This method involved initial solubilization and then precipitation of the enzyme with saturated concentrations of $(NH_4)_2SO_4$. A purification of 3 to 4-fold over the crude extract was achieved with this treatment.

The reaction was started by adding 0.1 ml of the enzyme preparation to 0.4 ml of the reaction mixture kept at 20 C. The reaction mixture consisted of 0.1 M PO_4 buffer, pH 7.0, 0.006 M carbamyl phosphate (CP), 0.09 M aspartate (ASP), and L and F synthetic seawater containing total NaCl concentrations ranging from 0.01 to 3.75 M. The reaction was stopped after 60 min by adding 1.0 ml of cold 0.1 N HCl in

absolute alcohol and stored in ice. Samples (0.75 ml) of the mixture was used for the determination of carbamyl aspartate (CA), the product of the reaction. Enzyme assay was based on the method of Koritz and Cohen (1954) for the colorimetric determination of CA. Carbamyl aspartate standards were made in L and F synthetic seawater containing total NaCl concentrations ranging from 0.01 to 3.45 M and treated in the same manner as the crude extract. Controls carried out for non-enzymatic reactions between CP and ASP produced no product (CA) when colorimetrically assayed.

CHAPTER IV

RESULTS

A. Selection and Isolation of NaCl-Tolerant Estuarine Bacteria.

1. Effect of Physical and Chemical Parameters on Bacterial Enumeration of Estuarine Water Samples.

In an ecological study, chemical and physical environmental parameters were investigated for the indigenous microflora in order to obtain optimal growth conditions. An experiment was designed to determine if temperature affected the aerobic heterotrophic population that developed from the Great Bay estuarine complex.

Estuarine water samples were collected December 24, 1973 (salinity, 20 ppt; temperature, 2.5 C; pH, 7.2) and August 28, 1974 (salinity, 28.5 ppt; temperature, 21.5 C; pH, 7.2) from the Jackson Estuarine Laboratory (JEL). Aliquots (0.1 ml) of the winter and summer water samples were enumerated on modified 2216E (Seven Seas) agar medium by spread plate technique after 30 days incubation at 4 C and 14 days incubation at 20, 37, and 50 C. The CFU/ml for the winter sample were: 1310, 2133, 837, and 10 at 4, 20, 37, and 50 C, respectively. The CFU/ml for the summer sample were: 1257, 2310, 1063, and 10 at 4, 20, 37, and 50 C, respectively. The percent error between triplicate plates of each water sample was less than 9. Maximum CFU/ml for

both water samples occurred at 20 C, and colony morphology and pigmentation were greatest at 20 C also. No seasonal selection of numbers or types of colonies was evident. The 20 C temperature was optimum and was used for subsequent enumeration studies.

Organic matter concentrations in modified 2216E agar medium were tested for optimum concentrations. Aliquots (0.1 ml) of the winter and summer water samples were enumerated on modified 2216E agar medium containing 0.02, 0.2, 1.0, and 2.0 % total organic matter after 14 days incubation at 20 C. The CFU/ml for the winter sample were 1993, 2243, 263, and 10 at 0.02, 0.2, 1.0, and 2.0 % organic matter, respectively. The CFU/ml for the summer sample were: 2000, 2363, 323, and 10 at 0.02, 0.2, 1.0, and 2.0 % organic matter, respectively. The percent error between triplicate plates of each water sample was less than 8. Maximum CFU/ml and colony diversity for both water samples occurred at 0.2 % organic matter concentration. The decreased CFU/ml at 1.0 and 2.0 % organic matter was due partly to confluent colonies and to an excess concentration of organic matter too concentrated for the majority of estuarine bacteria. The concentration of 0.2 % organic matter in modified 2216E agar medium was optimum and was employed for further investigations.

Since an estuary is a body of water in which fresh water mixes with seawater (Pritchard, 1967), salinity fluctuations were great (5-30 ppt). Since NaCl comprises 80-85 % of salinity, a determination of the effects of various

NaCl concentrations on the Great Bay estuarine bacterial population was undertaken. Aliquots (0.1 ml) of the winter and summer water samples were enumerated on modified MacLeod's Na-deficient agar medium containing total NaCl concentrations ranging from 0.0002 to 3.20 M. The plates were incubated at 20 C for 14 days. The percent error between triplicate plates of each water sample was less than 7.

Maximum CFU/ml occurred at 0.40 M NaCl for the winter sample and 0.30 M NaCl for the summer sample (Table 1). When the highest CFU/ml were taken as 100 %, nearly 50 % of the CFU/ml failed to develop at 1.00 M NaCl and only 1 % of the CFU/ml developed at 1.65 M NaCl. No colonies developed at 3.20 M NaCl. The occurrence of highest CFU/ml at NaCl concentrations (0.2 - 0.5 M) representative of those of the estuary (0.35 M), illustrated the stenohaline character of the bacterial population. The decrease in CFU/ml as NaCl concentrations deviated from those of the estuary indicated the oligodynamic effect NaCl had on the indigenous microflora. However, the occurrence of three CFU at 2.80 M NaCl suggested the presence in the estuary of halotolerant or halophilic bacteria.

The previous three experiments involving temperature, organic matter, and NaCl concentrations indicated that the cultural conditions yielding maximum CFU/ml were: 20 C, 0.2 % organic matter in modified 2216E (Seven Seas) agar medium containing 0.3 - 0.4 M NaCl.

2. Selective Effects of Supplemental NaCl Concentrations on the Estuarine Bacterial Population.

To study further the selective effects of supplemental NaCl on the estuarine bacterial population as well as to confirm the existence of NaCl-tolerant bacteria, the following experiment was performed. Estuarine water samples were collected aseptically at JEL from October 29, 1973, to November 30, 1974. For each sample, salinity, temperature, and pH were recorded. The seasonal fluctuations in these three parameters are shown in Fig. 1. Aliquots (0.1 ml) of the water samples were enumerated on modified 2216E (Seven Seas) agar medium with supplemental NaCl concentrations ranging from 0.0 to 3.05 M by spread plate technique after 14 days incubation at 20 C. Unsupplemented modified 2216E agar medium contained 0.30 M NaCl.

When the CFU/ml which developed on unsupplemented modified 2216E agar medium were used as comparison, CFU/ml dropped steadily when up to 1.0 M NaCl was added (Table 2). Generally, less than 20 % of the colonies developed at 1.0 M supplemental NaCl. At 1.85 M supplemental NaCl, less than 1 % of the CFU/ml developed. Single colonies developed at 2.25 and 2.65 M supplemental NaCl (recorded as 0 %) whereas no colonies developed at 3.05 M supplemental NaCl. The decrease in CFU/ml as NaCl concentration increased was consistent throughout the sampling period from October, 1973, to November, 1974 (Fig. 2). The occurrence of CFU's at 2.25 M NaCl (greater than 6-fold the average estuarine NaCl

concentration of 0.35 M) confirmed the existence of NaCl-tolerant estuarine bacteria. The nearly 50 % reduction in CFU/ml at 0.65 M supplemental NaCl demonstrated the toxic and selective effects that supplemental NaCl concentrations greater than those of the estuary have on the predominantly stenohaline estuarine bacterial population.

To assess further the effects of supplemental NaCl on the bacterial population, the data in Table 2 was investigated on the basis of pigmented and nonpigmented colonies. Pigmented colonies were those possessing a distinct color, such as, white, red, yellow, green, black, or orange; whereas nonpigmented colonies were beige.

Maximum pigmentation (34 %) occurred on modified 2216E agar medium (Table 3). Pigmentation dropped off to a minimum of 1 % as supplemental NaCl concentration increased to 1.45 M. No pigmented colonies occurred above 1.45 M supplemental NaCl. Supplemental NaCl concentrations approximately 4-5 fold those of the estuary produced greater than a 90 % decrease in colony pigmentation, further supporting the observation that the estuarine bacterial population was stenohaline.

The selective effects of supplemental NaCl on the estuarine bacterial population are presented photographically in Fig. 3. Maximum CFU/ml and diversity of colony types occurred in unsupplemented 2216E medium (0.30 M NaCl). CFU/ml and diversity decreased as supplemental NaCl concentration increased. At 2.60 M total NaCl (2.30 M supplemental NaCl) only two nonpigmented (beige) colonies developed.

Since nonpigmented (beige) colonies were seen exclusively at the highest NaCl concentrations that supported growth, an investigation to determine the taxonomy of the selected bacterial cells was undertaken. Microscopic examination performed on wet mounts and gram-stained cells showed that all cells were Gram-negative, nonmotile, rod-shaped, measuring 1.5-2.0 x 3.0-5.0 µm. The similarities in morphology and gram-stain reaction suggested a selection of a single halotolerant or extreme halophilic bacterial species.

An additional plate technique was employed to further substantiate the selective effects of supplemental NaCl on the estuarine bacterial population. The technique, replica plating, was designed to allow for the detection and isolation of organisms tolerant to the NaCl stress (Lederberg and Lederberg, 1952). Aliquots (0.1 ml) of water samples collected at JEL on November 25, 1973, April 30, 1974, July 19, 1974, and October 12, 1974, were enumerated on modified 2216E (Seven Seas) agar medium after 14 days incubation at 20 C. These plates were replica-plated subsequently on modified 2216E agar medium supplemented with NaCl concentrations ranging from 0.25 to 2.30 M. Following incubation at 20 C for 14 days, the plates containing 2.30 M supplemented NaCl were replica-plated on unsupplemented modified 2216E agar medium and incubated at 20 C for 14 days.

Maximum CFU and colony types developed on the master plates containing 0.30 M NaCl. CFU and diversity decreased

until no more than two beige colonies developed on 2.60 M NaCl-medium. However, when the 2.60 M NaCl-medium was replica-plated to unsupplemented NaCl-plates, CFU and diversity were similar to the master plates. The taxonomy of the bacterial cells examined from the highest NaCl-supplemented plates were similar to those of other NaCl-tolerant bacterial cells. However, cells from colonies on the master plates that corresponded to NaCl-tolerant colonies at superimposable sites on the highest NaCl-supplemented plates were 0.5 x 1.5 μm, rod-shaped, gram-negative, and motile. The replica plate technique demonstrated the stenohaline response of the estuarine bacterial population but confirmed the existence of NaCl-tolerant bacteria. The technique showed, also, the reversible effect of NaCl stress on the population. The occurrence of small, motile, rod-shaped bacterial cells on unsupplemented medium showed that NaCl stress altered cell morphology and motility.

3. Selective Effects of Other Supplemental Salts on the Estuarine Bacterial Population.

To determine if the response of the bacterial population to NaCl was specific, their response to supplemental salts other than NaCl was investigated. Aliquots (0.1 ml) of estuarine water samples collected at JEL on March 12, 1974, and August 21, 1974, were enumerated after 14 days incubation at 20 C on modified 2216E (Seven Seas) agar medium and the medium supplemented with one of the following salts: $NaNO_3$,

Na_2SO_4, KNO_3, KCl, $MgSO_4$, $CaSO_4$, or LiCl in concentrations ranging from 0.30 to 3.25 M.

When the CFU/ml which developed from the March water sample on unsupplemented modified 2216E medium were used as comparison, CFU/ml dropped steadily when up to 1.0 M salt was added (Table 4). With the exception of Na_2SO_4, 30 % or less of the colonies developed at 1.0 M supplemental salt. Less than 20 % of the colonies developed at supplemental salt concentrations greater than 2.1 M. No colonies developed at 3.25 M supplemental salts. Taxonomy of the bacterial cells from the most salt-tolerant colonies was similar to that for NaCl-tolerant bacteria. The data was supported by the August water sample (Table 5). Li and NO_3 were used at concentrations orders of magnitude greater than those of the estuary (Sverdrup et al., 1942). The data indicated that the survival of the estuarine bacterial population in the presence of other salts was similar to the response to supplemental NaCl. Salt stress was not NaCl specific. Salt-tolerant bacterial cells, similar to NaCl-tolerant cells, were obtained.

B. Identification of NaCl-Tolerant Bacterial Species.

Supplemental NaCl and other salts selected consistently for taxonomically similar bacterial cells. Diagnostic tests were conducted to determine the identity of these bacteria. Except where noted, tests were performed at 20 C either statically or shaken at 200 rpm in modified 2216E medium prepared with 26 ppt L and F synthetic seawater with

supplemental NaCl concentrations ranging from 0.0 to 3.45 M. In connection with the replica-plate technique, parent bacterial colonies which developed on modified 2216E agar medium and which corresponded to NaCl-tolerant colonies at superimposable sites on NaCl-supplemented medium were picked to aid in the identification of the halotolerant bacterial species. Bacteria cultured in modified 2216E medium were rod-shaped, 0.5 - 0.7 x 1.5 - 1.8 μm, varying to 1.5 - 2.0 x 3.0 - 5.0 μm in the medium supplemented with 3.45 M NaCl (Figs. 4-10). Cells occurred singly or in pairs (Figs. 4B, 4C, 4D), were motile with polar monotrichous flagellum at supplemental NaCl concentrations from 0.0 to 0.90 M (Figs. 8A, 8B, 9A, 10B) varying to nonmotile with loss of flagellum at supplemental NaCl concentrations greater than 1.30 M (Figs. 8C-F, 9B, 9C, 9D, 10C, 10D). No growth took place in distilled water medium. The cells were gram-negative, had no capsules (Indian ink stain), and spores (malachite green stain) were absent.

Colonies were nonpigmented, varying from 2-3 mm diameter, circular, smooth, raised on modified 2216E agar medium, on Extract Agar (BBL) prepared with distilled water, and on Bacto-Marine Agar 2216 (Difco) prepared with distilled water. On modified 2216E agar medium supplemented with NaCl concentrations ranging from 2.15 to 3.45 M, colonies were reduced to 1-2 mm diameter. No diffusible fluorescent pigments were produced.

Organic growth factors were not required. The culture was nutritionally versatile, able to use glucose, ribose, Na-acetate, Na-lactate, and Na-citrate as sole sources of carbon, unable to utilize glycerol, cellulose, or poly-β-hydroxybutyrate as sole carbon sources. The cells did not accumulate poly-β-hydroxybutyrate. The cells were able to use NH_4 and NO_3 as sole nitrogen sources but were unable to use creatinine and creatine as sole nitrogen and/or carbon sources. The cells were unable to use nicotine or thiotone for growth and unable to fix molecular nitrogen. Acid was produced from glucose, lactose, sucrose, galactose, fructose, mannose, maltose, dextrin, salacin, ribose, and sorbitol. No acid was produced from glycerol, rhamnose, xylose, raffinose, mannitol, arabinose, or inositol. Metabolism was always respiratory, never fermentative. The cells produced no gas or alkaline reactions. Gelatin, starch, urea, or poly-β-hydroxybutyrate were not hydrolyzed. Nitrate was denitrified weakly. Litmus milk and brom cresol milk remained unchanged. Polyethylene sorbitan monooleate (Tween 80) was hydrolyzed indicating extracellular lipase production. Arginine dihydrolase, lysine decarboxylase, and phosphatase were produced. Indole, H_2S, or acetylmethylcarbinol were not produced. The cells were sensitive to tetracycline (30 µg), streptomycin (10 µg), chloromycetin (30 µg), polymyxin (30 µg), and erythromycin (30 µg); they were insensitive to penicillin (10 units), trisulfa (10 µg), and 0/129 Vibriostat, pteridine, (30 µg). The cells were

obligately aerobic with an optimal temperature 20-25 C and temperature range 4-37 C. The optimal pH was 6.5-8.0, and the pH range 4.5-8.5. Catalase and oxidase were positive.

Since phenetic studies were dependent largely upon cultural conditions and involved only a fraction of the bacterial genome, up to 20 %, basepair and polynucleotide sequence homology studies were performed to assist in classifying and determining the similarity between the small, motile, bacterium and the large, nonmotile, NaCl-tolerant bacterium. The guanine plus cytosine (GC) molar percent of the DNA extracted from the NaCl-tolerant bacterium grown in modified 2216E medium and medium supplemented with 1.70, 2.60, and 3.45 M NaCl was 63.27 $\pm$ 0.01 (by buoyant density) and 63.18 $\pm$ 1.02 (by thermal melting) (Table 6). Thus, the organism grown in unsupplemented and NaCl-supplemented medium was a pure culture which was stable genetically.

DNA-DNA hybridizations were performed on the NaCl-tolerant bacterium grown in modified 2216E medium and the medium supplemented with 2.60 M NaCl. When the amount of hybridization which occurred between ^{32}P -DNA and unlabelled DNA from the bacterium grown at the same NaCl concentration was used as comparison, greater than 87 % hybridization occurred between the DNA extracted from the small, motile bacterium grown in unsupplemented medium and the DNA extracted from the large, nonmotile bacterium grown in 2.60 M supplemented-NaCl medium (Table 7). Since two DNA strands are considered alike if the hybridization is 75 % or greater

(Miller, 1972), the DNA extracted from the halotolerant bacterium grown in unsupplemented and NaCl-supplemented medium was similar.

The diagnostic tests indicated that supplemental NaCl selected consistently for a taxonomically similar halotolerant bacterium. This bacterium was gram-negative, rod-shaped, catalase and oxidase positive, 1.5 - 2.0 x 3.0 - 5.0 μm obligately aerobic, with a GC molar percent of 63. The organism was genetically stable and free of contamination. When grown in unsupplemented medium, the bacterium demonstrated similar characteristics as when grown in NaCl-supplemented medium, except that it was smaller and motile. On the basis of the phenetic and genetic tests, the bacterium corresponded to the description for the genus Pseudomonas as presented in the 8th edition of Bergey's Manual of Determinative Bacteriology (1974) and in A Guide to the Identification of the Genera of Bacteria (Skerman, 1967). The bacterium demonstrated a tolerance to 3.45 M supplemental NaCl. In accordance with the International Code of Nomenclature (1975), the bacterium was described as a new species, Pseudomonas halodurans. The species was not similar to other species of Pseudomonas listed. The species name complied with Recommendation 12C of The Code as it described a property of the species--the ability to tolerate high concentrations of NaCl.

C. Physiological Responses of P. halodurans to NaCl.

1. Growth Response to Salinity Variations.

Since P. halodurans was isolated from estuarine water in which salinity fluctuations occurred, a determination of salinity variations on the growth of P. halodurans in modified 2216E medium was undertaken. Modified 2216E medium with adjusted salinities was entered in 100 ml amounts into 250 ml Erlenmeyer flasks, sterilized, and inoculated with 1.0 ml of P. halodurans washed twice in L and F synthetic seawater made isotonic to 26 ppt L and F seawater with 1.097 M glycerol. Flasks were incubated at 20 C and 200 rpm. After hourly intervals, growth was measured as optical density at 420 nm.

P. halodurans grew optimally at a salinity of 28 ppt (Table 8). A high salinity of the Great Bay estuary during 1973 - 1975 was 28 ppt. Growth in 21 and 35 ppt salinity medium was suboptimal. No growth occurred at 0, 3.5, 245, or 350 ppt salinity over a period of 168 h. Thus, P. halodurans developed optimally in culture at estuarine salinities. Total salinities of greater than 175 ppt prevented growth indicating that NaCl was not as inhibitory as other salts in seawater since 3.45 M supplemental NaCl allowed growth in modified 2216E medium.

2. Major Ion Requirements of P. halodurans.

Although Na and Cl are the most abundant ions in seawater, other major ions such as Mg, K, Ca, SO_4, and Br have significant effects on marine bacteria (MacLeod, 1968). In order to study ionic effects on P. halodurans, specific

inorganic salts were deleted from or reduced in L and F synthetic seawater where glucose was the sole energy source in the medium. Equimolar glycerol (1.097 M) was used to substitute for deleted or reduced inorganic salts (MacLeod and Onofrey, 1956). Pre-sterilized test tubes containing 5 ml of sterile medium with pH adjusted with 6 N NH_4OH were inoculated with 0.05 ml of washed cell suspension of P. halodurans. The test tubes were incubated at 20 C and 200 rpm and observed turbidimetrically.

Optimal growth occurred at NaCl concentrations of 100 to 500 mM (Table 9). These salinities represented the range of NaCl concentrations of the estuary. Growth was suboptimal at NaCl concentrations below 100 mM. No growth occurred in the absence of NaCl in the medium. Thus, P. halodurans demonstrated an obligate Na requirement above 10 mM. Autolysis occurred in cultures incubated longer than 24 h as indicated by decreasing turbidity.

P. halodurans did not grow in the absence of either Na salts or NaCl deleted from L and F synthetic seawater (Table 10, Tubes 1 and 2). A minimum of 35 mM NaCl was needed for growth (Tube 4).

No growth occurred in the absence of $MgCl_2$ or in the presence of 4 mM $MgCl_2$ (Table 10, Tubes 9 and 10). A minimum of 8 mM $MgCl_2$ was needed for growth (Tube 11).

No growth occurred in the absence of $CaCl_2$ or in the presence of 1.5 mM $CaCl_2$ (Table 10, Tubes 13 and 14). A minimum of 3 mM $CaCl_2$ was needed for growth (Tube 15).

Although P. halodurans required KCl for growth (Table 10, Tubes 16 and 20,)it did not require $SrCl_2$, H_3BO_3, and KBr (Tubes 17, 18 and 19).

Thus, P. halodurans had an obligate requirement above 35 mM NaCl, 8 mM $MgCl_2$, 3 mM $CaCl_2$, and 4 mM KCl for growth. These concentrations were approximately 0.1, 0.16, 0.3, and 0.4 times, respectively, their estuarine concentrations.

To determine further NaCl, KCl, $MgCl_2$, and $CaCl_2$ concentrations that produced optimal growth of P. halodurans, as well as to determine the interactions between Na and K and Mg and Ca, the following investigation was undertaken. Five ml of sterile glucose seawater medium prepared with synthetic seawater in which either the NaCl concentration ranged from 0 to 500 mM, the KCl concentration from 0 to 100 mM, the $MgCl_2$ concentration from 0 to 100 mM and $CaCl_2$ concentrations from 0 to 100 mM were added to presterilized test tubes. The osmotic pressure was adjusted with 1.097 M glycerol as required. Each tube was inoculated with 0.05 ml of the washed P. halodurans cell suspension. The tubes were incubated at 20 C and 200 rpm.

Optimal growth of P. halodurans occurred at NaCl concentrations of 100 to 500 mM in the presence of 10 and 100 mM KCl (Table 11). Growth was suboptimal at NaCl concentrations below 100 mM in the presence of 10 and 100 mM KCl. No growth occurred in the absence of either NaCl or KCl after 72 h. No sparing effect of K for Na occurred. Optimal growth occurred at NaCl (100 - 500 mM) and KCl

concentrations (10 mM) representative of the concentrations of these two cations in the estuary. When KCl concentrations were 0 to 10 mM in 2 mM increments, and 50, 90, and 100 mM, optimal growth of P. halodurans occurred between 10 and 100 mM in the presence of 200 and 500 mM NaCl (Table 12). Growth was suboptimal at KCl concentrations lower than 10 mM and at 50 mM NaCl. Some sparing of Na for K was noted at 4 and 6 mM KCl in the presence of 50 and 200 mM NaCl. Thus, optimal growth of P. halodurans occurred at KCl (10 mM) and NaCl concentrations (200 - 500 mM) similar to those of the estuary. Incubation beyond 24 h did not increase growth.

In connection with other major ions, $MgCl_2$ and $CaCl_2$ were utilized to determine optimal concentrations for growth of P. halodurans. When $MgCl_2$ concentrations of 1, 10, 25, 50, and 100 mM and $CaCl_2$ concentrations of 10 and 100 mM were used, optimal growth of P. halodurans occurred at 50 mM $MgCl_2$ and 10 and 100 mM $CaCl_2$ (Table 13). Growth was suboptimal at $MgCl_2$ concentrations other than 50 mM, in the presence of 10 and 100 mM $CaCl_2$. No growth occurred in the absence of either $MgCl_2$ or $CaCl_2$ after 72 h. No sparing effect of Ca for Mg was evident. Optimal growth of P. halodurans occurred at $MgCl_2$ (50 mM) and $CaCl_2$ concentrations (10 mM) similar to those of the estuary.

When $CaCl_2$ concentrations were 0, 1, 3, 5, 10, 25, and 50 mM and $MgCl_2$ concentrations were 0.0, 50, and 100 mM, optimal growth of P. halodurans occurred at 10 mM $CaCl_2$ and 50 and 100 mM $MgCl_2$ (Table 14). Suboptimal growth was evident

at $CaCl_2$ concentrations other than 10 mM in the presence of 50 and 100 mM $MgCl_2$. No sparing effect of Mg for Ca was evident. Thus, optimal growth of P. halodurans occurred at $CaCl_2$ (10 mM) and $MgCl_2$ concentrations (50 mM) representative of estuarine concentrations.

P. halodurans demonstrated an obligate requirement for Na, K, Mg, and Ca. A study was undertaken to determine if similar obligate requirements by the bacterium for Cl, SO_4, and Br, the major anions in seawater, existed. Five ml of sterile glucose seawater medium prepared with L and F synthetic seawater in which the anion concentrations, (0 to 200 mM) were added as their Na salts were Cl, Br, or SO_4, respectively, added to pre-sterilized test tubes. Each tube was inoculated with 0.05 ml of the washed P. halodurans cell suspension. The tubes were incubated at 20 C and 200 rpm and observed turbidimetrically.

Optimal growth of P. halodurans occurred at 200 mM Cl, Br, or SO_4 (Table 15). Suboptimal growth occurred below 200 mM of each anion concentration. No growth was evident in the absence of these anions in the medium after 72 h. Since mole for mole, Br and SO_4 were as effective as Cl in promoting growth of P. halodurans, the organism demonstrated a requirement for an anion which was satisfied by Cl, Br, or SO_4 alone. The amount of anion required for growth was (1 mM).

P. halodurans in culture demonstrated an obligate requirement for the most abundant cations in seawater (Na,

Mg, K, Ca). The optimal concentrations of these ions for growth were similar to their concentration in the estuary from which the organism was isolated. The lack of sparing between Na and K, and Mg and Ca indicated that the function of these ions in the growth of P. halodurans was not interchangeable.

3. Growth Response of P. halodurans to Supplemental NaCl.

Since supplemental NaCl concentrations up to 2.60 M selected consistently for a halotolerant bacterium identified as P. halodurans, a study to determine the effects of supplemental NaCl on a pure culture of the bacterium was undertaken. Modified 2216E medium and the medium supplemented with 0.0, 0.90, 1.70, 2.60, 3.45, and 4.30 M NaCl as 100 ml volumes in 250 ml Erlenmeyer flasks were sterilized and inoculated with 1.0 ml of the standard inoculum. Flasks were incubated at 20 C and 200 rpm and sampled periodically for 144 h for optical density and observed microscopically for morphological changes.

The presence of increasing concentrations of NaCl resulted in prolonging the lag phase (Fig. 11). The lag phase of P. halodurans in the presence of 1.20 M NaCl was extended 1.5 h, but the slope of the curve and the maximum optical density were similar to that in modified 2216E medium. As the total NaCl concentration increased to 2.00, 2.90, and 3.75 M, the lag phase was extended 7, 12, and 57 h, respectively. No growth occurred over a period of 144 h in 4.60 M NaCl. The growth rate during the logarithmic phase of

growth was decreased with each increase in NaCl concentration greater than 1.20 M.

Since the light transmittance depends upon the size and shape of the particles, as well as their numbers, the values show the initiation of growth, general shape of the curve, and the time of attainment of maximum optical density at each NaCl concentration and not necessarily absolute changes in cell numbers. To determine the relation between cell numbers and dry weight, and optical density, the following experiment was performed. Aliquots (1.0 ml) of the cultures were removed periodically and diluted in 9.0 ml of L and F synthetic seawater made isotonic to the growth medium with NaCl (w/v). Diluted cell suspensions (0.1 ml) were enumerated by spread plate after 10 days incubation at 20 C on modified 2216E medium of the corresponding NaCl concentration as the growth culture. Direct counts using a Petroff-Hauser counting chamber were performed on 1.0 ml aliquots of the cultures fixed with 10 % formalin and counted microscopically. Dry weights of 1.0 ml aliquots of twice washed cells were determined after drying at 100 C for 48 h in tared aluminum weighing pans.

At 0.0, 0.90, 1.70, and 2.60 M supplemental NaCl, CFU/ml, direct count, and dry weight increases coincided with increases in the optical density of the culture (Table 16). The correlation between CFU/ml and direct count indicated greater than 90 % cell viability and reproducibility under increased NaCl stress. At 3.45 M supplemental NaCl

only plate counts were determined at the stationary phase. CFU/ml ranged from 6.5 - 8.5 x 10^8. The data indicated that optical density was a reliable index for determining the growth of P. halodurans. The specific growth rate was maximal (0.325) in unsupplemented modified 2216E medium and decreased steadily as NaCl concentrations increased (Table 17). At 3.45 M supplemental NaCl, the growth rate was decreased approximately an order of magnitude. Concurrent with a decrease in the growth rate and optical density was a nearly 20-fold increase in the lag phase compared to the unsupplemented medium. The data was representative of results obtained when P. halodurans was stressed with similar NaCl concentrations in the glucose seawater medium (Table 17). Although optimal growth of P. halodurans occurred at a NaCl concentration approximating that of the estuary, the organism was able to tolerate and reproduce at NaCl concentrations greater than 12-fold estuarine concentrations indicating its halotolerance. Phase contrast microscopy of late logarithmic phase P. halodurans cells indicated an increase in cell volume as NaCl concentrations increased. Actively motile cells in unsupplemented (0.30 M NaCl) and 1.20 M NaCl medium were 0.5 - 0.7 x 1.5 - 1.7 μm and 0.7 - 1.0 x 1.5 - 2.0 μm, respectively (Figs. 6A and 6B). As NaCl concentration increased to 2.00, 2.90, and 3.75 M, cells were nonmotile and measured 1.2 - 1.5 x 2.0 - 3.0 μm, 1.5 - 2.0 x 3.0 - 4.0 μm, and 2.0 - 2.5 x 3.5 - 5.0 μm, respectively (Figs. 6C, 6D, and 6E). Cell volume at 3.75 M NaCl was 21 volumes

larger than cells in unsupplemented modified 2216E medium. Since cell size and motility did not change markedly during the growth cycle of P. halodurans in modified 2216E medium (Fig. 4), morphological and motility alterations were due to the supplemental medium NaCl.

Transmission and scanning electron microscopy of late logarithmic cells indicated that P. halodurans at 0.30 and 1.20 M NaCl was monotrichous polarly flagellated (Figs. 8A, 8B, 9A, and 10A and B). At NaCl concentrations from 1.60 to 3.75 M, a flagellum was absent (Figs. 8C - F, 9B - D, and 10C and 10D). The loss of motility at NaCl concentrations approximately 3-4 fold those of the estuary indicated that flagellation was more sensitive to NaCl stress than growth and reproduction.

To pursue further the effects of supplemental NaCl on P. halodurans cells, 2.60 M NaCl was added to 250 ml aliquot of an early logarithmic phase culture (OD=0.25) from modified 2216E. The 2.60 M supplemental NaCl provided an adequate stress within 72 h. Flasks incubated at 20 C and 200 rpm had 1.0 ml aliquots removed at 12, 24, 48, and 72 h after NaCl addition.

Phase contrast microscopy indicated that greater than 90 % of P. halodurans cells were approximately 3 volumes larger than unstressed cells and motility inhibited after 12 h NaCl addition (Fig. 7A). Cell volume increased to about 5, 10, and 20 volumes larger than unstressed cells after 24, 48, and 72 h, respectively (Figs. 7B - D). Thus, the effect

of supplemental NaCl on P. halodurans cells was relatively slow, requiring 12 h for morphological and between 12 and 24 h for motility alterations to occur.

Since supplemental NaCl altered the morphology and motility of P. halodurans, an investigation to determine the effect of suboptimal NaCl concentrations on the morphology and motility of the bacterium was undertaken. Sterile modified 2216E medium as 100 ml in 250 ml Erlenmeyer flasks with total NaCl concentrations of 0.01, 0.05, 0.10, and 0.30 M was inoculated with 1.0 ml of the twice washed standard inoculum. Flasks were incubated at 20 C and 200 rpm. P. halodurans cells were observed microscopically and photographed at the late logarithmic growth phase.

In the presence of 0.01 M NaCl, cells exhibited limited motility and were 0.8 - 1.2 x 1.5 - 2.0 μm (Fig. 5A). Cells were actively motile and varied from oval-shaped to rod-shaped as NaCl concentrations increased from 0.5 to 0.30 M NaCl (Figs. 5B - D). The inhibition in motility and the swollen shape of the cells at the suboptimal NaCl concentrations of 0.03, 0.15, and 0.3 times that in modified 2216E medium indicated P. halodurans was adapted optimally to these concentrations. These hypotonic solutions suggested some problems in osmoregulation by P. halodurans.

4. Growth Response of P. halodurans to Glycerol and Sucrose.

Supplemental NaCl concentrations up to 3.45 M altered motility and morphology of P. halodurans cells while decreasing the specific growth rate and maximum optical density and

increasing the lag phase. To determine if these changes were due to osmotic or ionic stress, the following experiment was performed. Sterile modified 2216E medium (100 ml) in 250 ml Erlenmeyer flasks was supplemented with either glycerol or sucrose in concentrations ranging from 0.0 to 3.5 M. Glycerol and sucrose were used because they were nonpenetrating, nonionic solutes. The medium was inoculated with 1.0 ml of the standard inoculum. Flasks were incubated at 20 C and 200 rpm and sampled periodically for 72 h for optical density and observed microscopically for morphological change.

The specific growth rate, lag phase, and maximum optical density for P. halodurans cells exposed to 1.0 to 3.5 M glycerol or sucrose were very similar to untreated cells (Table 18). No morphological or motility alterations occurred at supplemented glycerol or sucrose concentrations. Thus, the effect of supplemental NaCl on P. halodurans was ionic primarily and not osmotic.

5. Growth Response of P. halodurans to Salts Other Than NaCl.

Estuarine water is composed of ions other than NaCl. An experiment was designed to determine how ions other than NaCl affected the morphology, motility, and growth of P. halodurans. Sterile modified 2216E medium as 100 ml in 250 ml Erlenmeyer flasks were supplemented with one of the following salts: $NaNO_3$, Na_2SO_4, KCl, KNO_3, LiCl, NH_4Cl, $MgCl_2$, or $CaCl_2$ in concentrations ranging from 0.0 to 3.45 M. The medium was inoculated with 1.0 ml of the standard inoculum and incubated at 20 C and 200 rpm. The medium was sampled periodically for

144 h for optical density and observed microscopically for morphological alteration. Ions such as Li, NO_3, and NH_4 are present in ppb amounts in seawater.

The presence of increasing concentrations of the salts resulted in a decrease in the specific growth rate and maximum optical density and an increase in the lag phase and cell size (Table 19). With the exception of LiCl and NH_4Cl, 0.90 M supplemental salts had little effect on the growth of P. halodurans compared to the unstressed cells. At 0.90 M LiCl and NH_4Cl, the specific growth rate was decreased approximately 50 and 80 %, respectively, compared to untreated cells. As all salt concentrations increased above 0.90 M, the specific growth rate and maximum optical density decreased from 40 to 90 % while the lag phase was extended from 5 to 11-fold compared to unstressed cells. With the exception of LiCl and NH_4Cl, supplemental salt concentrations increased the cell size 15 - 20 volumes compared to untreated cells. Motility was not evident above 0.90 M. The alterations in morphology, motility, and growth of P. halodurans in varied salt solutions indicated that the effects of supplemental NaCl on the organism were not ion specific.

6. Adaptation of P. halodurans to Supplemental NaCl.

The ability of an organism on repeated subculture to become adapted to a stress is well known (Alexander, 1971). An experiment was conducted to determine if P. halodurans cells adapted to the ionic effects of supplemental NaCl. Sterile modified 2216E medium and the medium supplemented

with 3.45 M NaCl were inoculated with 1.0 ml of the standard inoculum. Late logarithmic phase P. halodurans cells from the medium were subcultured 4 times in modified 2216E medium containing the corresponding NaCl concentration as the initial growth medium and incubated at 20 C and 200 rpm. After the fourth transfer had reached the late logarithmic phase, 1.0 ml aliquots were removed and inoculated separately into 250 ml Erlenmeyer flasks containing sterile modified 2216E medium with supplemental NaCl concentrations ranging from 0.0 to 4.30 M. Flasks were incubated at 20 C and 200 rpm and sampled periodically for 144 h for optical density and observed microscopically for morphological changes.

Preculturing of P. halodurans in 0.30 or 3.45 M NaCl had little effect on the response to NaCl concentrations in the growth medium (Table 20). As NaCl concentrations increased to 3.45 M, the specific growth rate and maximum optical density decreased approximately an order of magnitude and 75 %, respectively, compared to the unsupplemented medium. The lag phase increased 20-fold compared to the unsupplemented medium. No growth occurred after 144 h in 4.30 M supplemented-NaCl medium. Concurrent with the effect on growth was an effect on morphology and motility. Regardless of precultured NaCl concentrations, as NaCl concentrations increased, cell size increased approximately 21 volumes while motility was lost above 0.90 M supplemental NaCl. Cells precultured in 3.45 M supplemented-NaCl medium were 2.0 - 2.5 x 3.0 - 5.0 μm and nonmotile. When placed in unsupplemented medium the cells

became actively motile and measured 0.6 - 0.8 x 1.5 - 1.8 µm within 8 h. P. halodurans cells tolerated but did not adapt to the increased ionic stress created by the supplemental NaCl.

To determine if each P. halodurans cell in the population was genetically stable in its response to various NaCl concentrations, the following investigation was undertaken. Aliquots (1.0 ml) of late logarithmic phase cells from the precultured modified 2216E medium and the medium supplemented with 3.45 M NaCl were removed and diluted in synthetic seawater made isotonic to the growth medium with NaCl (w/v). The dilutions were prepared to yield a low, easily countable number of colonies (20-120 CFU/ml). Aliquots (0.1 ml) of the diluted cell suspensions were enumerated after 10 days incubation at 20 C on modified 2216E agar medium with total NaCl concentrations ranging from 0.01 to 5.40 M.

Whether P. halodurans cells were precultured in modified 2216E medium or the medium supplemented with 3.45 M NaCl, the effects of fluctuating NaCl concentrations were very similar (Table 21). Maximal CFU/ml developed between 0.20 and 0.30 M NaCl. A progressive decrease in CFU/ml occurred at lower and higher NaCl concentrations up to 3.75 M. No growth occurred at 4.60 and 5.40 M NaCl.

When genetic stability was tested further by replica plating the initial plates to plates containing modified 2216E medium with the corresponding NaCl concentration, nearly every colony on the initial plate gave rise to a colony on the replica plate (Table 21). P. halodurans cells were

genetically stable to the increased ionic stress of supplemental NaCl. In conjunction with Table 20, P. halodurans demonstrated a readily reversible response to supplemental NaCl up to 3.45 M indicating a tolerance and not adaptation to the NaCl stress.

7. Cultural Stage of Growth of P. halodurans Versus the Effect of 2.60 M Supplemental NaCl.

The physiological age of a culture may be a determinant in the response of that culture to stress. This effect of physiological age may reflect differences in the content of lipid, proteins, nucleic acids, growth factors, intermediates of these groups, permeability properties, and inorganic ion constituents of the cells (Lamanna and Mallette, 1965).

The added stress in this experiment was 2.60 M NaCl added to aliquots of the culture during various stages in the life cycle. The change in population was followed by plate count. Sterile modified 2216E medium(100 ml)in 250 ml Erlenmeyer flasks were inoculated with 1.0 ml of the standard inoculum. Flasks were incubated at 20 C and 200 rpm. At 0, 1, 2, 4, and 8 h, 50 ml aliquots of the cultures were removed, placed in sterile 125 ml Erlenmeyer flasks, and 2.60 M NaCl added. At 0, 1, 3, 4, 8, 12, 16, 20, 36, and 48 h after NaCl addition, 0.1 ml aliquots of the subcultures growing at 20 C and 200 rpm were enumerated after 10 days incubation at 20 C on modified 2216E agar medium.

The control (broken line) underwent a normal sigmoidal curve; the first 2 h being lag phase, a logarithmic period

lasting 12 h, an abbreviated stationary phase to 16 h, and a subsequent decline phase until 48 h (Fig. 12). Addition of NaCl at 0 and 1 h showed slight increases in CFU after 16 h with major increases in CFU between 20 and 36 h. The increase at 36 h represented about 1 and 2 doublings for the 0 and 1 h cultures, respectively. Additions at 2 and 4 h produced significant bursts in CFU after 12 h finally yielding about 5 and 6 doublings, respectively, after 20 h. Addition at 8 h failed to produce a division in the subsequent 40 h.

The cell may need to accumulate some materials for division. If so, the cells appear to have accumulated this material at highest concentration by the end of the lag phase. If NaCl was added before or after this time, the cells were able to go through only one or two divisional cycles. The closer the addition of NaCl was to the end of the lag phase, the greater the population increased and the shorter the time needed to achieve this change.

8. Effect of Temperature and Organic Matter on Growth and NaCl Tolerance of P. halodurans.

The response of an organism to stress may also be dependent upon the cultural conditions at which the organism is grown. The response of P. halodurans to the increased ionic stress caused by supplemental NaCl was determined at one temperature and organic matter concentration. An experiment was designed to determine if alternate temperatures and organic matter concentrations affected the tolerance of P. halodurans to supplemental NaCl.

Sterile modified 2216E medium (100 ml) was prepared with total peptone and yeast extract concentrations of 0.02, 0.2, and 1.0 % and total NaCl concentrations ranging from 0.01 to 3.75 M. The medium in 250 ml Erlenmeyer flasks was inoculated with 1.0 ml of the standard inoculum. Flasks were incubated at 200 rpm at 5, 10, 15, 20, 25, 35, and 40 C. Optical density measurements were made periodically for 144 h at 15 to 40 C and 260 h at 5 and 10 C.

In the presence of 1.0 % organic matter in the medium, P. halodurans grew optimally, as determined by specific growth rate, at 0.30 M NaCl regardless of the incubation temperature (Fig. 13). Minimal growth occurred at 0.01 M NaCl for all temperatures. Growth rates declined steadily at higher NaCl concentrations than 0.30 M. The organism tolerated 2.90 M NaCl at 5, 10, 15, and 35 C, and 3.75 M NaCl at 20 and 25 C. No growth occurred at 40 C. The incubation temperature affected the NaCl tolerance of P. halodurans with maximal tolerance at 20 and 25 C.

When P. halodurans was grown at 0.2 % medium organic matter, optimal growth rates occurred at 0.30 M NaCl regardless of incubation temperature (Fig. 14). The organism tolerated 2.90 M NaCl at 5, 10, 15, and 35 C, and 3.75 M NaCl at 20 and 25 C. No growth occurred at 40 C. Although the response of P. halodurans to supplemental NaCl was not affected by 0.2 % medium organic matter compared to 1.0 % organic matter, incubation temperature did affect the halotolerance of the bacterium.

At 0.02 % medium organic matter, P. halodurans tolerated 2.10 M NaCl at 5 C and 2.90 M NaCl at 10 to 35 C (Fig. 15). Although the large increments of NaCl concentrations made exact determinations of the NaCl response of P. halodurans to cultural conditions difficult, incubation temperature and organic matter concentration in the medium did affect the halotolerance of the bacterium.

To demonstrate the NaCl response of P. halodurans, the maximum optical density of the culture grown at 20 C and 200 rpm at NaCl concentrations ranging from 0.0 to 4.30 M in modified 2216E medium containing 0.2 % organic matter was plotted (Fig. 16). Maximum growth occurred between 0.30 and 1.20 M NaCl and declined steadily at lower and higher NaCl concentrations. The ability of P. halodurans to reproduce over a NaCl concentration range of 0.01 to 3.75 M demonstrated the broad NaCl response and halotolerance of the organism.

9. Growth Response of Marine Bacteria Other Than P. halodurans to Supplemental NaCl.

To determine if other marine bacteria tolerated the increased ionic stress of supplemental NaCl concentrations many folds greater than those of the estuary, sterile modified 2216E medium as 100 ml in 250 ml Erlenmeyer flasks was supplemented with NaCl concentrations ranging from 0.0 to 3.45 M. The medium was inoculated with 1.0 ml of twice washed, late logarithmic bacterial cultures as follows: P. halodurans, Arthrobacter marinus, P. cuprodurans, Pseudomonas sp. 130, and Alteromonas marinopraesens. In addition,

selected pigmented colonies isolated from the ecological studies, designed; C-1 (P. halodurans), C-2 (red pigmented), C-3 (yellow pigmented), C-4 (white pigmented), and C-5 (orange pigmented) were investigated for NaCl tolerance. The flasks were incubated at 20 C and 200 rpm. They were sampled periodically for 144 h for optical density and observed microscopically for morphological changes.

P. halodurans tolerated 3.45 M supplemental NaCl (Table 22). A. marinus and P. cuprodurans tolerated 2.60 M supplemental NaCl. The specific growth rate decreased greater than an order of magnitude and the maximum optical densities decreased 60 and 85 %, respectively, compared to the unsupplemented medium. The lag phase was extended to 72 and 80 h, respectively. Cells became nonmotile at 1.70 M supplemented NaCl for both cultures and increased 5 volumes compared to unstressed cells. Pseudomonas sp. 130 and A. marinopraesens were the least adaptable bacteria to supplemental NaCl, tolerating 1.70 M NaCl, with little morphological alteration. The lag phases were extended to 67 and 122 h, respectively. P. halodurans, among the five identified bacteria, exhibited the greatest tolerance to NaCl.

The P. halodurans culture (C-1) was the same as used in the previous table. Estuarine isolate, C-2, was the least adaptable bacterium, tolerating only 0.90 M supplemental NaCl, with little morphological change (Table 23). Isolates C-3 and C-4 tolerated 1.70 M supplemental NaCl, with the lag phases extended to 10.5 and 18.5 h, respectively. Estuarine

isolate, C-5, was the most adaptable bacterium, tolerating 2.60 M supplemental NaCl, with no morphological change. The lag phase was extended from 3.5 to 26.5 h. The specific growth rate decreased nearly an order of magnitude compared to the unsupplemented medium. P. halodurans was the most adaptable of the five estuarine bacteria to NaCl, exhibiting the least effect and greatest tolerance up to 3.45 M supplemental NaCl.

10. Effect of NaCl on Oxygen Utilization by P. halodurans.

Growth and reproduction of P. halodurans was reduced as the ionic concentration of the medium deviated from optimal. Since growth is a direct function of respiration, an investigation was undertaken to determine the effect of NaCl concentrations on the respiration of P. halodurans. Sterile modified 2216E medium as 100 ml unsupplemented and supplemented with 1.70 and 2.60 M NaCl in 250 ml Erlenmeyer flasks was inoculated with 1.0 ml of the standard inoculum. Upon attaining the late logarithmic growth phase at 20 C and 200 rpm, the cells were harvested and prepared for respiration studies. The cells were placed in respiration flasks containing modified 2216E medium with total NaCl concentrations ranging from 0.01 to 5.45 M. Respiration readings were taken every 10 min for 3 h with initial and final dry weights determined. When grown in unsupplemented medium, respiration, measured as oxygen utilization by P. halodurans was maximal (640 μlitersO_2/mg dry weight) in the presence of 0.20, 0.25, and 0.30 M NaCl after 180 min (Fig. 17). Although respiration

decreased progressively at lower and higher NaCl concentrations, the slopes of the lines at each NaCl concentration indicated that the rate of oxygen utilization was uniform. The effect of NaCl concentration on respiration was very similar to its effect on growth of P. halodurans. The respiratory activity was optimal at NaCl concentrations representative of the range of NaCl concentrations of the estuary. Further, the occurrence of respiratory activity at 4.60 M NaCl-a concentration at which the organism failed to grow-indicated that the respiratory processes, or other less sensitive physiological functions, were more halotolerant than the replication processes.

Respiration by P. halodurans cells grown in 1.70 M NaCl-supplemented medium was maximal (420 μlitersO_2/mg dry weight) at 0.20 and 0.30 M NaCl (Fig. 18). Although respiration decreased steadily at higher NaCl concentrations, the rate of oxygen utilization was uniform at each NaCl concentration. The decrease in respiration as NaCl concentrations increased above 0.30 M was consistent for untreated and NaCl-precultured cells. Preculturing cells in 1.70 M NaCl-supplemented medium suppressed the respiration of P. halodurans. The consistent decrease in respiration at NaCl concentrations greater than 0.30 M indicated that adaptation to 1.70 M NaCl did not occur.

Cells precultured at a higher NaCl stress (2.60 M supplemented NaCl) demonstrated maximal respiration (210 μ litersO_2/mg dry weight) at 0.20 and 0.30 M NaCl (Fig. 19). Respiration decreased steadily at higher NaCl concentrations.

At each NaCl concentration, the rate of oxygen utilization was uniform. Absolute amounts of oxygen utilization by 2.60 and 1.70 M NaCl-precultured P. halodurans cells studied in NaCl concentrations from 0.20 to 4.60 M were reduced 70 and 45 %, respectively, compared to amounts for untreated cells.

The reduction in growth of P. halodurans by NaCl concentrations not representative of estuarine concentrations (0.2 -0.35M) was due partially or entirely to the reduction in respiratory rates. The consistent decrease in oxygen utilization in NaCl-precultured cells indicated that supplemental NaCl concentrations did not select for P. halodurans cells.

11. Respiratory Deficiency of P. halodurans Induced by NaCl.

Triphenyltetrazolium chloride (TTC) has been used to detect respiratory deficiency in yeast (Lindegren et al., 1958). The testing of respiratory deficiency was scored by the inability to reduce TTC within 1 h; the colonies remain white, while respiratory sufficient colonies turn red from the reduced formazan.

NaCl concentrations different from those of the estuary (0.35 M) reduced the respiration of P. halodurans. An experiment was designed to determine if the reduced respiration caused respiratory deficiency in the cells. Sterile modified 2216E medium as 25 ml in 125 ml Erlenmeyer flasks was inoculated with 0.25 ml of the standard inoculum. After attaining the late logarithmic growth phase at 20 C and 200 rpm, cells were harvested and twice washed in

NaCl-deleted synthetic seawater made isotonic to the growth medium with 1.097 M glycerol. After dilution of the cells in the isotonic wash solution, 0.1 ml aliquots were spread on modified 2216E agar medium with total NaCl concentrations ranging from 0.01 to 3.75 M. Plates were incubated for 10 days at 20 C. The colonies were overlayed with 0.1 % TTC in either 1.5 % agar or 10 % gelatin and re-incubated an additional 1 h at 20 C. The gelatin which was kept at 37 C was used to determine if the 50 C temperature at which the agar was kept was detrimental to respiration.

The colonies produced a red color within 1 h, indicating respiratory sufficiency (Table 24). Colonies grown at 0.20 to 0.30 M NaCl produced a red color within 6 min. These salinities represented NaCl concentrations of the estuary. Time to produce a red color increased at lower and higher NaCl concentrations to a maximum of 57 min at 3.75 M NaCl. Although NaCl concentrations beyond the range of the estuary caused reduced respiration in P. halodurans cells, these concentrations did not induce absolute respiratory deficiencies in the cells.

12. Thunberg Studies on P. halodurans.

Although O_2 uptake serves as a suitable method for measuring the terminal reaction of aerobic respiration, it frequently is desirable to measure electron transport at a point before the terminal oxidation, or to measure the activity of systems which do not transport electrons to O_2. Thunberg emphasized the role of hydrogen activation in

respiratory processes and measured anaerobic dye reduction by hydrogen (electrons) as an index of their activation by dehydrogenase enzymes (Umbreit et al., 1964). To investigate further the effects of NaCl on respiratory activity by P. halodurans, the Thunberg technique was employed.

To study the electron transport system of P. halodurans, three oxidation-reduction (O/R) dyes were used; methylene blue (MB), and dichloroindophenol (DCIP) since they accepted electrons at the flavoprotein-quinone level of electron transport because of their comparable O/R potentials (Lardy, 1949), and TTC to determine where the dye accepts electrons. With the use of these O/R dyes, it was possible to determine if the prolonged TTC reduction times were reflective of changes in the electron transport system at or before the flavoprotein-quinone level, or at the cytochrome level.

Sterile modified 2216E medium as 100 ml with total NaCl concentrations ranging from 0.01 to 3.75 M in 250 ml Erlenmeyer flasks was inoculated with 1.0 ml of the standard inoculum. Upon attaining the late logarithmic phase of growth at 20 C and 200 rpm, the cells were harvested, thrice washed, and resuspended in 5 ml synthetic seawater made isotonic to the growth medium with NaCl (w/v). Thunberg tubes were prepared and optical density performed at 1 min intervals for 36 min on the tubes incubated at 20 C against blanks containing the respective dyes and components including a formalized cell suspension.

Maximum reduction of TTC by P. halodurans occurred at 0.10 and 0.30 M NaCl (Fig. 20). A progressive delay and decrease in reduction occurred at 2.00, 2.90, 0.01 and 3.75 M, respectively. The rate of reduction was decreased also at these four NaCl concentrations. Dehydrogenase activity of P. halodurans was maximum at NaCl concentrations representative of estuarine concentrations from which the organism was isolated.

Maximum reduction of DCIP occurred at 0.10 and 0.30 M NaCl (Fig. 21). In the presence of 2.00, 2.90, 0.01 and 3.75 M NaCl, a progressive delay and decrease in reduction as well as a decrease in reduction rate occurred. DCIP reduction indicated that dehydrogenase activity was maximum at NaCl concentrations representative of the estuary.

The reduction of MB by P. halodurans was maximum at 0.10 and 0.30 M NaCl (Fig. 22). Dehydrogenase activity was maximum at estuarine NaCl concentrations but decreased as NaCl concentrations differed from those of the estuary.

The effect of NaCl concentrations on the dehydrogenase activity of P. halodurans indicated that the reduction in growth of the organism by NaCl concentrations different than those of the estuary may be due in part to the reduction in dehydrogenase activity. The decrease in reduction rates of TTC, DCIP, and MB suggested that NaCl affected the electron transport system at or before the flavoprotein-quinone level.

13. Qualitative Measurements of the Cytochrome Components of P. halodurans.

The increased ionic strength caused by supplemental NaCl in the medium decreased the activity of dehydrogenase enzymes of P. halodurans. To determine if supplemental NaCl affected enzymes involved with the terminal transfer of electrons in aerobic respiration, the cytochromes of P. halodurans unstressed and stressed with supplemental NaCl were studied.

Sterile modified 2216E medium as 500 ml and the medium supplemented with 2.60 M NaCl in one-liter Erlenmeyer flasks were inoculated with 5 ml of the standard inoculum. Upon attaining the late logarithmic phase at 20 C and 200 rpm, the cells were harvested and prepared for cytochrome analysis. Optical density measurements of the cell extracts containing the cytochromes were made between 400 and 650 nm.

The absorption spectra obtained from the reduced whole cell extracts containing the cytochromes of P. halodurans grown in the unsupplemented medium (A, B, C) and 2.60 M NaCl-supplemented medium (A', B', C') were similar (Fig. 23). Both spectra of cytochromes showed A and A' peaks (alpha peaks) at 635 nm, B and B' peaks (beta peaks) at 597 and 590 nm, respectively, and C and C' peaks (gamma or Soret peaks) at 415 and 425 nm, respectively. The occurrence of alpha peaks at 635 nm suggested strongly the presence of the Pseudomonas blue protein and the cytochromes a which show absorption maxima in this region (**Lehninger, 1975**).

The difference spectra obtained from the oxidized and reduced whole cell extracts of P. halodurans grown in the unsupplemented medium (A, B, C, D) and 2.60 M NaCl-supplemented medium (A', B', C', D') were similar (Fig. 24). Both spectra of cytochromes showed alpha peaks (A, A'), beta peaks (B, B'), troughs between 455 and 465 nm (C, C'), and gamma (Soret) peaks (D, D').

The similarities in curves from both absorption and difference spectra of cytochromes from unstressed and stressed P. halodurans cells indicated that an increase in the ionic strength of the medium approximately 10-fold that of the estuary did not alter the cytochromes of the bacterium.

14. Intracellular Na and K Concentrations of P. halodurans in the Presence of 0.30, 1.60, and 2.90 M NaCl.

The ability of supplemental NaCl concentrations in modified 2216E medium to affect the activity of dehydrogenase enzymes of P. halodurans suggested an increase in the intracellular ionic concentration and strength of the cytoplasm. Since an increase in the intracellular concentrations of Na and especially K has been related to halophilism and halotolerance of halphilic and nonhalophilic bacteria, respectively (Christian and Waltho, 1962), an investigation to determine the intracellular concentrations of these ions in P. halodurans cells under NaCl stress was undertaken.

Sterile modified 2216E medium as 1,000 ml and the medium supplemented with 1.30 and 2.60 M NaCl in two-liter,

acid-washed Erlenmeyer flasks were inoculated with 10 ml of the standard inoculum and incubated at 20 C and 200 rpm. Aliquots (200 ml) of the medium were removed at the early, mid-, and late logarithmic as well as the stationary phase of growth. The cells were harvested by centrifugation and analyzed for intracellular Na and K by atomic absorption spectrophotometry.

When P. halodurans cells were grown in modified 2216E medium containing 345 mM Na and 12 mM K, intracellular Na and K concentrations were highest at the early logarithmic growth phase (Table 25). Intracellular Na varied from 1.38 times greater than medium Na at the early logarithmic phase to approximately equivalent Na at the stationary phase. Intracellular K was approximately double the medium K at the early logarithmic phase and decreased to 1.71 times the medium K at the stationary phase. Intracellular fluid volume was highest at the early logarithmic phase (1.99 ml/g dry weight) but remained relatively constant throughout the growth cycle. The ratio of intracellular Na to intracellular K indicated that K was accumulated to a greater extent than Na. P. halodurans exhibited intracellular Na and K at concentrations equal to or greater than those of its environment with the concentrations of these ions dependent upon the physiological age of the culture.

In the presence of 1.30 M supplemental NaCl, P. halodurans cells had the highest intracellular concentrations of Na and K at the early logarithmic growth phase (Table 26).

In modified 2216E containing 1659 mM Na and 12 mM K, intracellular Na varied from 70 to 50 % medium Na and intracellular K from nearly 18 to 11-fold medium K as the cells progressed from the early logarithmic to the stationary growth phase. Medium Na was 137-140 times greater than medium K, but intracellular Na was only approximately 6 times greater than intracellular K owing to the relatively greater accumulation of K than Na. Intracellular fluid volume was highest at the early logarithmic phase (5.27 ml/g dry weight) and represented greater than a 2.5-fold increase compared to the maximum intracellular fluid volume for untreated cells. P. halodurans cells did not exclude Na and K when grown in 1.30 M supplemental NaCl, accumulating nearly 2.5 and 8.5 times the intracellular Na and K, respectively, compared to untreated cells. This increased intracellular ionic concentration was concurrent with an increased intracellular fluid volume compared to untreated cells.

When P. halodurans cells were grown in 2.60 M NaCl-supplemented modified 2216E medium containing 2920 mM Na and 12 mM K, the intracellular Na and K concentrations were highest at the early logarithmic growth phase (Table 27). Intracellular Na varied from slightly less than 50 to 36 % medium Na and intracellular K varied from 32 to nearly 20-fold medium K as the cells progressed from the early logarithmic to the stationary phase. Medium Na was nearly 250 times medium K while intracellular Na was less than 4 times intracellular K owing to the relatively greater accumulation of K

than Na. Intracellular fluid volume was highest at the early logarithmic phase (8.49 ml/g dry weight) and represented greater than a 4-fold increase compared to untreated cells. Intracellular Na and K concentrations increased nearly 3 and 16-fold compared to intracellular Na and K concentrations for untreated cells.

The increase in intracellular Na and K as medium NaCl concentration increased indicated that these ions were not excluded from the cells. Intracellular Na concentrations were dependent upon medium Na concentrations suggesting that Na entered the cells passively. If so, the cells appeared to have a limit to the amount of intracellular Na, since less than 50 % of medium Na was intracellular for cells grown at 2.90 M NaCl. On the other hand, the increased intracellular K concentration, up to 32-fold greater than medium K, at 2.90 M NaCl suggested an active uptake of K. Thus, the relation between K accumulation and halotolerance may be applied to P. halodurans. The concurrent increase in intracellular fluid volume with increased intracellular Na and K concentrations and subsequent increased intracellular ionic concentration of the cytoplasm suggested that the increased volume in cell size at supplemental NaCl concentrations greater than 12-fold those of the estuary was due primarily to H_2O uptake.

To get an idea of the rapidity at which NaCl-stressed cells take up Na and K from the medium, the following experiment was performed. Aliquots (200 ml) from the early

logarithmic phase culture in modified 2216E medium from the preceding experiment were removed to sterile, acid-washed 500 ml Erlenmeyer flasks. Prior to the addition of 2.60 M NaCl (time zero), 50 ml aliquots were removed and analyzed for intracellular Na and K. The flasks were then incubated at 20 C and 200 rpm. At 24, 48, and 72 h after the NaCl addition, 50 ml aliquots were removed and analyzed for intracellular Na and K. The Na concentration in the unsupplemented medium was 350 mM and the K concentration was 12 mM. The 2.60 M NaCl-supplemented medium contained 2922 mM Na and 12 mM K.

The addition of 2.60 M supplemental NaCl resulted in an increase in the intracellular fluid volume from 1.87 to 4.21 ml/g dry weight, intracellular Na concentration from 458 to 2146 mM, and intracellular K concentration from 24.4 to 288 mM after 24 h (Table 28). Intracellular fluid volume increased to a maximum of 8.53 ml/g dry weight after 72 h. Although intracellular Na concentration increased, the ratio of intracellular Na to medium Na decreased from 1.31 to 0.73, 24 h after the addition of NaCl. In contrast, the ratio of intracellular K to medium K increased greater than an order of magnitude to 24.0, 24 h after NaCl addition. Although the maximum increase in intracellular fluid volume was slow, requiring 72 h, maximum accumulation of Na and K occurred within 24 h.

15. Accumulation of ^{22}Na by Intact Cells and Cell Envelopes of P. halodurans Under NaCl Stress.

P. halodurans accumulated from 483 to 1382 mM Na, dependent upon medium NaCl concentration and the age of the culture. To determine the percent of Na that was associated with the cell and the cell envelope, the following experiment utilizing radioactive $^{22}NaCl$ was performed. Sterile modified 2216E medium as 500 ml and the medium supplemented with 1.30 and 2.60 M NaCl in one-liter, acid-washed Erlenmeyer flasks were supplemented with $^{22}NaCl$ to initial concentrations of 0.006 μCi/ml (2.22×10^{-2} nM), 0.03 μCi/ml (11.1×10^{-2} nM), and 0.06 μCi/ml (22.2×10^{-2} nM), respectively. The three media were inoculated with 5 ml of the standard inoculum used in the previous experiment. Flasks were incubated at 20 C and 200 rpm with 10 ml aliquots removed one and two h after inoculation and 100 ml aliquots removed at the early, mid-, late logarithmic, and stationary growth phases. The cells were harvested by centrifugation, and analyzed for cell-associated ^{22}Na. Cell envelopes were extracted from a portion of the cells and analyzed for cell envelope-associated ^{22}Na.

Cell-associated ^{22}Na for P. halodurans cells in unsupplemented modified 2216E medium varied from 4.35 to slightly more than 1 % of the medium ^{22}Na concentration as the physiological age of the culture progressed from early logarithmic to stationary phase (Table 29). The percent of cell-associated ^{22}Na to medium ^{22}Na concentration varied from 5.1 to 0.66 % and 8.8 to 1.2 % for cells grown in 1.30

and 2.60 M NaCl supplemented-medium, respectively. The amount of ^{22}Na associated with the cell envelopes of P. halodurans was less than 2 % the medium ^{22}Na concentration. However, the amount of ^{22}Na associated with the cell envelopes varied from 22 to nearly 13 % the amount associated with the cells. Thus, P. halodurans cells accumulated the majority of ^{22}Na interior to the cell envelope. The cell envelope may have a limited number of binding sites that are synthesized rapidly during the early logarithmic growth phase of P. halodurans. Limited numbers of binding sites would allow for only a small percent of the total ^{22}Na in the medium to bind rapidly to the cell envelope.

If Na-binding sites were limited, then the addition of supplemental NaCl and $^{22}NaCl$ to an early logarithmic phase culture should result in little if any additional cell envelope-associated ^{22}Na.

The remaining 80 ml of ^{22}Na-associated P. halodurans cells grown in modified 2216E medium from the preceding experiment was supplemented with 2.60 M NaCl and 0.39 µCi/ml (to maintain the relation between labelled and unlabelled Na in the medium). Prior to the addition of supplemental NaCl and $^{22}NaCl$ (time zero), 15 ml aliquots were removed and analyzed for cell-associated and cell envelope-associated ^{22}Na. At 24, 48, and 72 h after the addition of labelled and unlabelled Na at 20 C and 200 rpm, 15 ml aliquots were removed and analyzed for cell-associated and cell envelope-associated ^{22}Na.

The addition of supplemental $^{22}NaCl$ to an early logarithmic P. halodurans culture did not increase the percent of cell envelope-associated ^{22}Na to cell-associated ^{22}Na (Table 30). Percent cell envelope-associated ^{22}Na declined from 20 % at time zero to 15 % 72 h after $^{22}NaCl$ addition. Maximum cell-associated ^{22}Na occurred 24 h after $^{22}NaCl$ addition. P. halodurans cell envelopes probably contained a limited number of Na-binding sites that were occupied rapidly during the early logarithmic growth phase. If so, the remaining ^{22}Na associated with the cells was located interior to the cell envelope.

When the cell-associated ^{22}Na data from the previous experiments were related to the dry weight of the cells, maximum cell pellet-associated ^{22}Na/mg dry weight occurred during the early logarithmic growth phase regardless of the NaCl concentration of the medium (Fig. 25). Values decreased steeply as the cultures aged. When an early logarithmic phase culture was stressed with 2.60 M NaCl and 0.39 µCi/ml $^{22}NaCl$ (indicated by arrow in Fig. 25), cell-pellet associated ^{22}Na/mg dry weight was maximum at a point corresponding to the extended early logarithmic phase of the NaCl-stressed culture. Regardless of medium NaCl and whether studied as cell-associated ^{22}Na or cell pellet-associated ^{22}Na/mg dry weight, ^{22}Na uptake occurred rapidly during the early logarithmic growth phase of P. halodurans.

D. Biochemical Responses of P. halodurans to Supplemental NaCl Concentrations Ranging From 0.0 to 3.45 M.

1. Carbon, Nitrogen, Phosphorus Ratios of P. halodurans in the Presence of NaCl Stress.

The ratio of carbon (C), nitrogen (N), and phosphorus (P) vary throughout the normal growth cycle (Brock, 1970). When 2.60 M supplemental NaCl was added to a growing culture of P. halodurans (OD=0.25), the optical density remained constant for 24 h before increasing to a maximum of 0.80 after 72 h. An experiment was undertaken to determine cellular activity during the time of the unchanged optical density.

Sterile modified 2216E medium as 150 ml in 250 ml Erlenmeyer flasks was inoculated with 1.5 ml of the standard inoculum. Upon incubating at 20 C and 200 rpm for three h, the medium was subdivided into three 50 ml aliquots. Aliquot one was stressed with 2.60 M supplemental NaCl, aliquot two was stressed with 4.30 M supplemental NaCl, a prohibitive concentration for growth, and aliquot three was untreated. Periodically, samples were removed for enumeration on modified 2216E agar medium. Cells were harvested by centrifugation at 10,000 x g for 15 min at 4 C, washed twice in synthetic seawater, and brought to an optical density of approximately 0.5 at 420 nm. From this cell suspension, carbon was determined by the method of Menzel and Vaccaro (1964) using CO_2 as a standard, nitrogen by micro-Kjeldahl (Jacobs, 1965) using $(NH_4)_2SO_4$ (Fisher) as a standard, and

phosphorus by the method of Allen (1940) using KH_2PO_4 (Fisher) as a standard.

As determined by CFU/ml, the control (0.30 M NaCl) underwent a sigmoidal growth curve; a 3 h lag phase, a 7 h logarithmic phase, and a declining phase (Fig. 26A). The addition of 2.60 M NaCl resulted in a reduced rate of cell division up to 7 h after addition, then an accelerated rate for the following 26 h with a subsequent stabilization thereafter to 48 h. The addition of 4.30 M NaCl inhibited multiplication completely, with no growth occurring.

The C:P and N:P ratios of the control rose gradually, coincident with the early logarithmic phase, then increased nearly 2-fold the original levels, before dropping, coincident with the declining phase of growth (Fig. 26B). The addition of 2.60 M NaCl resulted in a steady increase in the C:P and N:P ratios to nearly 2-fold after 48 h compared to the C:P and N:P ratios at the time of NaCl addition. The addition of 4.30 M NaCl resulted in steady low C:P and N:P ratios, similar to that at the time of NaCl addition. The addition of 2.60 M NaCl repressed but did not arrest growth and multiplication of P. halodurans. The repressed cells began to multiply rapidly once the cells adjusted to the increased ionic concentration of the medium. In light of the increased intracellular Na and K concentrations at supplemental medium NaCl concentrations and the similarity in the C:P and N:P ratios of unstressed and NaCl-stressed, no ionic-induced metabolic imbalance occurred as P. halodurans adjusted to the 2.60 M NaCl stress.

2. DNA:RNA:Protein:Carbohydrate:Phospholipid Ratios of *P. halodurans* in the Presence of Supplemental NaCl.

The biochemical constituents of *P. halodurans* are composed, in part, of carbon, nitrogen, and phosphorus. An additional technique to study the effect between supplemental NaCl and growth and multiplication of the organism utilized determining the concentrations of the biochemical constituents: DNA, RNA, protein, carbohydrate, and phospholipid at various growth phases of the culture. An alteration in the biosynthesis of one or more of the biochemical constituents could assist in explaining the reduced growth of *P. halodurans* as supplemental medium NaCl concentration increased.

Duplicate, sterile modified 2216E medium as 100 ml with supplemental NaCl concentrations ranging from 0.0 to 3.45 M in 250 ml Erlenmeyer flasks were inoculated with 1.0 ml of the standard inoculum. Aliquots (25 ml) of the culture grown at 20 C and 200 rpm were removed at the early, mid-, late logarithmic, and stationary growth phases. Cells were washed in 25 ml of synthetic seawater made isotonic to the growth medium with NaCl (w/v) and analyzed for DNA, RNA, protein, carbohydrate, and phospholipid.

P. halodurans cells grown to the early logarithmic phase demonstrated the highest concentrations of DNA, RNA, protein, carbohydrate, and phospholipid in unsupplemented medium (Table 31). Biochemical constituent concentrations decreased steadily to approximately 20 % compared to unstressed-cell concentrations at 3.45 M supplemental NaCl. When the

biochemical constituent concentrations were compared to DNA, the ratios of DNA:RNA:protein:carbohydrate:phospholipid were unaltered by supplemental NaCl concentrations. Thus, the effect of supplemental NaCl concentrations up to 3.45 M on the early logarithmic growth of P. halodurans could not be attributed to the increased intracellular ionic concentrations of Na and K causing changes in the biosynthesis of the cell's biochemical constituents.

When P. halodurans cells were studied at the mid-logarithmic phase, the ratios of DNA:RNA:protein:carbohydrate:phospholipid were unchanged by supplemental NaCl concentrations to 3.45 M (Table 32). Thus, supplemental NaCl concentrations did not affect the biochemical constituent concentration of mid-logarithmic P. halodurans cells. The reduced growth rate of P. halodurans during the mid-logarithmic phase as supplemental medium NaCl concentration increased was not due to ionic-induced changes in the biosynthesis of any of the biochemical constituents.

Although the concentrations of the biochemical constituents were highest for P. halodurans cells in unsupplemented medium at late logarithmic phase, the ratios of DNA:RNA:protein:carbohydrate:phospholipid remained consistent as the supplemental NaCl concentration increased to 3.45 M (Table 33). Supplemental NaCl concentrations greater than 12-fold those of the estuary did not alter the biosynthesis of any of the biochemical constituents during the late logarithmic growth phase.

The ratios of DNA:RNA:protein:carbohydrate:phospholipid of stationary phase P. halodurans cells were not changed by supplemental NaCl concentrations in the medium (Table 34). Supplemental NaCl concentrations did not alter the ratio of the biochemical constituents of P. halodurans during the growth cycle. Thus, the ability of supplemental NaCl concentrations up to 12-fold gerater than those of the estuary to reduce growth and multiplication of P. halodurans was not due to ionic-induced alterations in the biosynthetic machinery necessary for DNA, RNA, protein, carbohydrate, and phospholipid synthesis.

3. The 220-320 nm Absorbance of Cultural Supernates From NaCl-Stressed P. halodurans Cells.

In sufficient concentration, an inhibitor of cell growth may accumulate at the cell surface and induce permeability changes in the cell membrane-cell wall complex (Salton, 1964). Since supplemental NaCl concentrations greater than 0.90 M inhibited growth of P. halodurans, an experiment was designed to determine if a correlation existed between the reduced growth of the organism and changes in the cell complex permeability. Rather than study permeability directly, the indirect assessment was made by leakage of ultraviolet (UV)-absorbing compounds from the interior of the cell to the medium.

Sterile modified 2216E medium as 100 ml and the medium supplemented with 1.70 and 3.45 M NaCl in 250 ml Erlenmeyer flasks were inoculated with 1.0 ml of the standard inoculum. Aliquots (50 ml) were removed at the early

logarithmic and stationary growth phases of the culture incubated at 20 C and 200 rpm. Samples (1.5 ml) of twice-centrifuged supernates were placed in matched quartz curvettes and the optical density read against the uninoculated medium as a reference in the range of 220-320 nm. The values plotted as stationary cultures were from cultures at maximum optical density.

The maximum absorbance curve occurred with the supernate from P. halodurans cells stressed with 3.45 M supplemental NaCl (Fig. 27). Stationary phase cultural supernates demonstrated consistently greater absorbance curves than their early logarithmic supernate counterparts. However, both 1.70 and 3.45 M supplemental NaCl early logarithmic supernates demonstrated more absorbance than the 0.30 M stationary phase supernate. The relationship between increased medium NaCl concentration and increased UV-absorbance in the corresponding NaCl-supplemented supernate suggested that the reduced growth of NaCl-stressed P. halodurans may be due in part to ionic alteration in cell permeability.

4. Quantitative and Qualitative Chemical Composition of P. halodurans Cell Envelope Under NaCl Stress.

To determine if the suggested permeability alteration was due to changes in the quantitative and/or qualitative chemical composition of the cell wall (cell envelope), the following experiment was performed. The cell pellets from the previous experiment were resuspended in 25 ml of synthetic seawater made isotonic to the growth medium by the addition

of either 0.0, 1.70, or 3.45 M supplemental NaCl. Cell envelopes were removed and washed to remove adherent cell membrane and cytoplasmic material.

a. Chemical Composition of NaCl-Stressed Cell Envelopes.

Aliquots (10 ml) of the cell envelope suspensions were analyzed for DNA, RNA, protein, carbohydrate, phospholipid, nitrogen, and phosphorus. Samples (3 ml) were removed from the 10 ml aliquot and hydrolyzed by the method of Salton (1953). Reducing substance was determined by the anthrone test of Seifer et al. (1950) using glucose (Fisher)as a standard. Hexosamine was determined by the method of Cessi and Piliego (1960) using glucosamine (Aldrich) as a standard. Total lipid was determined by the method of Salton (1953). Prior to extraction, duplicate 0.5 ml samples of the 10 ml aliquot of cell envelope suspensions were placed in tared aluminum weighing pans and incubated at 110 C for 36 h for dry weight determinations.

The major chemical constituent of P. halodurans cell envelopes was protein which was greater than 73 % by dry weight of the cell envelope (Table 35). Carbohydrate (21 %), total lipid (6 %) and nitrogen (12 %) were next most abundant constituents. No quantitative or qualitative alterations occurred in the P. halodurans cell envelopes stressed with 1.70 and 3.45 M supplemental NaCl compared to unstressed cell envelopes. Thus, there was no ionic-induced alteration in the chemical composition of NaCl-stressed cell envelopes.

b. Amino Acid Content of NaCl-Stressed Cell Envelopes.

Since quantitative differences in protein concentration of cell envelopes did not occur, qualitative differences were studied by examining the amino acid content of P. halodurans cell envelopes under NaCl stress.

Aliquots (10 ml) of the cell envelope suspensions were hydrolyzed by the method of Hill (1965) and the amino acid composition determined with a Beckman Model 1200 Amino Acid Analyzer.

Regardless of the medium NaCl concentration, the most abundant amino acids were aspartic acid, alanine, glycine, and leucine (Table 36). Supplemental NaCl of 1.70 and 3.45 M did not induce quantitative or qualitative alterations in the amino acid content of P. halodurans cell envelopes.

c. Phospholipid Content of NaCl-Stressed Cell Envelopes.

Since the phospholipid composition of P. halodurans cell envelopes under NaCl stress was unchanged compared to unstress cell envelopes, an investigation was undertaken to determine if NaCl-induced qualitative alterations occurred.

Aliquots (5 ml) of P. halodurans cell envelope suspensions were analyzed for phospholipids using thin-layer chromatography (Folch et al., 1957).

Regardless of the medium NaCl concentration (0.30, 2.00, or 3.75 M) comparative tracings of thin-layer chromatograms were qualitatively similar (Fig. 28). The tracings showed 6 distinct spots; one unidentified and 5 representing

the following phospholipids: phosphatidyl-choline (Rf=0.18), phosphatidyl-serine (Rf=0.34), phosphatidyl-ethanolamine (Rf=0.65), diphosphatidyl-glycerol (Rf=0.84), and phosphatidic acid (Rf=0.94). The size and location of the spots indicated no ionic-induced alteration in the amount or type of phospholipids in NaCl-stressed cell envelopes compared to unstressed cell envelopes.

d. Thin Sections of Intact Cells and Cell Envelopes Under NaCl Stress.

Transmission electron microscopy and biochemical analyses of cell envelopes of P. halodurans indicated no NaCl-induced alteration in cell envelope integrity. To demonstrate further the integrity of cell envelopes under NaCl stress, 1 ml of the cell envelope suspensions were thin-sectioned and examined with the Philips 200 Transmission Electron Microscope. The micrographs of thin-sections of cell envelopes were then compared to micrographs of thin-sectioned whole cells grown at similar NaCl concentrations to the similar physiological phase .

Thin sections of extracted P. halodurans cell envelopes demonstrated no visual alteration at 2.00 and 3.75 M medium NaCl compared to unstressed cell envelopes (Fig. 29). The cell envelopes were intact and maintained an oval to rod shape. Supplemental medium NaCl concentrations did not alter the integrity of P. halodurans cell envelopes.

Similarly, thin sections of whole cells demonstrated no visual alteration in the integrity of cell envelopes under NaCl stress compared to unstressed cell envelopes (Fig. 30).

Although the increased ionic strength of the growth medium created by supplemental NaCl concentrations altered the cell membrane-cell wall of P. halodurans, the lack of biochemical and mechanical alteration in the cell envelopes suggested that the tolerance of the organism to NaCl concentrations greater than 12-fold those of the estuary was due in part to the mechanical and biochemical maintenance of the cell envelope.

5. Aspartate Transcarbamylase Activity of P. halodurans As Affected by NaCl.

The intracellular ionic concentration in P. halodurans cells increased as medium NaCl concentration increased. An investigation was undertaken to determine the effect of the increased intracellular ionic strength on enzymatic activity. NaCl concentrations below those of the estuary were utilized to study further a wide range of NaCl concentrations on the growth of P. halodurans. The enzyme studied, aspartate transcarbamylase (ATCase), was chosen since it was relatively easy to extract and represented the first enzyme in the biosynthesis of the pyrimidine, uridine-5'-phosphate. The enzyme has been studied extensively as a model of regulatory enzymes (Lehninger, 1975).

Sterile modified 2216E medium as 500 ml and the medium supplemented with 2.60 M NaCl in one-liter Erlenmeyer flasks

were inoculated with 5 ml of the standard inoculum. Upon reaching the stationary growth phase at 20 C and 200 rpm, the cells were harvested and sonicated to release ATCase. The specific activity of the enzyme, purified by the method of Kaplan et al. (1967), was determined colorimetrically by the production of the end-product, carbamyl aspartate (CA) (Koritz and Cohen, 1954), in the presence of NaCl concentrations ranging from 0.01 to 3.75 M. The cells were precultured in 2.60 M supplemental NaCl to determine the ability of ATCase to become adapted to increased intracellular ionic concentrations.

ATCase specific activity was maximum between 0.30 and 0.70 M NaCl regardless of the precultured-NaCl concentration (Fig. 31). Maximum specific activity varied from approximately 210 - 250 μM CA/min/mg protein. Specific activity was suboptimal at higher and lower NaCl concentrations. ATCase activity was approximately 10-15 % maximum activity at 3.75 M NaCl. The enzyme was stenohaline in relation to optimum activity, but was euryhaline in the ability to demonstrate activity over a wide range of NaCl concentrations. The similarities between enzymatic activity and growth of P. halodurans, i.e., optimum growth or activity in a narrow NaCl range while halotolerant to a much wider NaCl spectrum, suggested that the effect of NaCl concentrations different from those of the estuary on P. halodurans was partially explainable by ionic-induced alterations in the enzymatic activity of the organism. Similar to whole cells

and respiratory studies, adaptation of ATCase to supplemental NaCl concentrations and increased intracellular ionic strengths in the cytoplasm did not occur.

CHAPTER V

DISCUSSION

The bacterial population of the Great Bay estuarine complex was investigated for tolerance to NaCl addition up to 3.05 M (Table 2). The population was primarily stenohaline since the addition of 1.0 M NaCl allowed less than 20 % of the colonies to develop when compared to maximum CFU/ml which developed on modified 2216E medium. Less than 1 % of the CFU/ml developed in 1.85 M supplemental NaCl compared to modified 2216E medium. ZoBell (1946) found that doubling the salinity of seawater either by the addition of NaCl or sea salts reduced by 25 to 50 % the number of marine bacteria which developed. He found that less than 10 % of marine bacteria grew in seawater media to which 2.0 M NaCl had been added. Other examples of the stenohaline character of marine and estuarine bacteria were shown by Brown and Turner (1963), Tyler et al. (1960), and Shah and deSa (1964) who found that 1.2 to 2.0 M supplemental NaCl inhibited greater than 60 % of the isolates. Thus, salt tolerance of marine and estuarine bacteria was limited, and it has been pointed out that halotolerance was more common in terrestrial bacteria (MacLeod, 1965).

However, halotolerant and extreme halotolerant bacteria do exist in the marine and estuarine environments (Forsyth et al., 1971). These investigators found that of 69 bacteria isolated from the intertidal waters at St. Andrews,

N.B., nearly one-third multiplied at 3.0 to 4.0 M supplemental NaCl. These extreme halotolerant isolates were primarily gram-negative rods. The occurrence of colony forming units from Great Bay estuarine water samples at 1.85 M supplemental NaCl (Table 2) indicated that halotolerant gram-negative bacteria existed in the estuary. With an increase in medium NaCl concentration, the viable count of various estuarine bacteria increased until a certain concentration between 0.30-0.40 M NaCl, varying with species, had been reached; thereafter this count dropped progressively with further NaCl increase, up to 2.80 M total NaCl (Tables 1, 2, 3; Figs. 2, 3). A selective process was involved; some bacteria were better able to survive and reproduce under NaCl stress. The persistence of one halotolerant bacterial colony from the Great Bay estuary at NaCl concentrations above 2.0 M was indicative of the selectivity. This bacterium was identified as a new species, P. halodurans, since it tolerated up to 3.75 M NaCl in pure culture.

Due to its requirement for at least 7 ppt seawater (Table 8) and 10 mM NaCl (Table 9), P. halodurans was considered a marine bacterium since it corresponded to the definitions of marine bacteria set forth by ZoBell and Upham (1944) and MacLeod (1965, 1968). Additionally, the bacterium demonstrated requirements for 4 mM KCl (Table 10), 8 mM $MgCl_2$ (Table 10), and 3 mM $CaCl_2$ (Table 10). These ionic requirements were 0.03, 0.4, 0.16, 0.3 times, respectively, their estuarine concentrations (Sillén, 1961). The requirements

of P. halodurans for Mg and Ca corresponded to results for growth of Pseudomonas aeruginosa (Eagon, 1969; Asbell and Eagon, 1966). D'Aoust and Kushner (1971) reported that Na and Mg were essential for the growth of a red psychrophilic marine bacterium. MacLeod and Onofrey (1957a, b) found that Na, Mg, K, Ca, and Cl were required for the growth of A. marinopraesens. The amount and rate of growth of A. marinopraesens was dependent upon the Na, K, and Cl concentration in the medium, with optimal growth at seawater concentrations (0.47 M Na, 0.05 M Mg, 0.01 M K, 0.01 M Ca, and 0.55 M Cl, Sillén, 1961). Similarly, the amount and rate of growth of P. halodurans was dependent upon the concentration of medium Na, Mg, K, Ca, and Cl with optimal growth at concentrations representative of those of the estuary.

P. halodurans grew over a range of NaCl concentrations from 10 mM to 3.75 M (Fig. 16). However, NaCl concentrations isotonic to the organism (0.30 - 1.20 M) produced best growth. The wide range of NaCl concentrations tolerated by P. halodurans encompassed concentrations indicative of marine, moderate halophilic, and extreme halophilic bacteria (Larsen, 1962).

Other marine bacteria have not demonstrated the halotolerance of P. halodurans. Three unidentified marine bacteria investigated by MacLeod and Onofrey (1957a) did not grow in the presence of 1.0 M NaCl in the medium. Of 15 marine bacteria studied by Tyler et al. (1960), all grew at 0.8 M NaCl, 9 grew at 1.4 M NaCl, and none grew at 2.6 M NaCl. Among the marine and estuarine bacteria tested in this

dissertation, none grew above 2.60 M NaCl, except P. halodurans (Tables 22 and 23).

However, many terrestrial, nonhalophilic bacteria tolerate NaCl concentrations greater than those tolerated by marine bacteria (Larsen, 1962). Staphylococcus aureus tolerated, but did not require for growth, up to 2.5 M NaCl (Scott, 1957). Salmonella oranienburg tolerated 2.0 M NaCl (Christian, 1955). Aerobic sporeforming bacteria and many micrococci tolerated 2.5 to 4.3 M NaCl (Larsen, 1962). Thus, P. halodurans tolerated NaCl concentrations similar to those tolerated by some terrestrial, nonhalophilic bacteria but generally higher than those tolerated by most marine bacteria.

The cell morphology of P. halodurans varied with the NaCl concentration in the medium. At NaCl concentrations hypotonic to P. halodurans, 0.01, 0.05, and 0.10 M, cells were motile, oval to rod-shaped, measuring 1.0 x 2.0 μ m (Fig. 5A, B C). At NaCl concentrations isotonic to those of the estuary, cells were motile, rod-shaped, measuring 0.5 x 1.7 μm (Fig. 5D, 6A, 8A, and 9A). The increase in cell volume at hypotonic NaCl concentrations was attributed to water uptake as dictated by the internal osmotic pressure exceeding the external osmotic pressure (Doetsch and Cook, 1973). As NaCl concentration increased above estuarine concentrations, the cells became swollen and motility inhibited (Fig. 6B and 8B). At NaCl concentrations above 1.20 M to 3.75 M, cell volumes increased up to 21 volumes larger than unstressed cells, measuring 2.0 - 2.5 x 4.0 - 5.0 μ m with a loss of

flagellum (Fig. 6C, C, E, 8C-F, and 9B, C, D). Cell volume increase took 48-72 h, whereas the loss of motility took 24 h, indicating that motility was more halosensitive than growth to 2.90 M NaCl (Fig. 7).

The physiology and morphology of P. halodurans was not affected when exposed to glycerol and sucrose, both nonionic compounds, at 1.0 to 3.5 M concentrations in modified 2216E medium. The osmotic pressure of modified 2216E medium (0.35 M NaCl) at 20 C was 0.364 g-mol/liter and increased to 4.382 g-mol/liter at 3.75 M NaCl (Handbook of Chemistry and Physics 54th ed., 1974). With glycerol added at concentrations of 1.0 to 3.5 M, the osmotic pressure increased from 0.603 to 2.814 g-mol/liter. The osmotic pressure for sucrose at 1.0 M was 0.947 g-mol/liter and increased to 1.406 g-mol/liter at 1.2 M. NaCl-induced osmotic pressures of 2.814 and 1.406 g-mol/liter corresponded to approximately 2.5 and 1.3 M NaCl, respectively. Since these NaCl concentrations altered the physiology and/or the morphology of P. halodurans while similar osmotic pressures created by glycerol (3.5 M) and sucrose (1.2 M) did not, the effects of NaCl on the organism were ionic and not osmotic. Further support that the effects on P. halodurans were ion-induced, was provided by major ions other than Na and Cl (i.e. K, Mg, Ca, and SO_4) which produced similar physiological and morphological alterations in the organism as NaCl (Table 19).

NaCl-induced morphological and motility alterations were found in other bacteria. Brown (1961) found that the marine pseudomonad NCMB 845 changed from sphere-shaped (1 to 2 μm diameter) or short elipsoid at the lowest NaCl concentration that showed growth (0.06 M) to short rods (0.5 x 1.2 μm) at 0.18 M NaCl. M. halodenitrificans demonstrated a swollen appearance at NaCl concentrations below 0.5 and above 3.5 M (Takahashi and Gibbons, 1959). Extreme halophilic bacteria (Halobacterium halobium, H. salinarium, and H. cutirubrum) grown in the range of 4.5 to 2.0 M NaCl exhibited morphological changes from long slender motile rods, through irregular club shapes, to nonmotile spheres as the NaCl concentration became hypotonic to the internal NaCl concentration (Mohr and Larsen, 1963). Doetsch and Cook (1973) noted that a number of Bacilli sp. showed a loss of flagellation and motility as the cells became plasmolyzed in a hypertonic NaCl solution (1.0 M).

Attempts to adapt P. halodurans cells to NaCl concentrations greater than 1.0 M failed (Tables 20 and 21). Further experiments indicated that the entire population of P. halodurans cells was halotolerant in response to NaCl (Table 21). Thus, P. halodurans was stable genetically to supplemental NaCl concentrations to 3.75 M (12-fold estuarine concentrations).

The NaCl response of nonhalophilic and obligate halophilic bacteria was genetically stable also (Ingram, 1957 ; Larsen, 1962; MacLeod, 1965). Forsyth and Kushner (1970)

found that cultures of M. halodenitrificans and V. costicolus, two moderate halophilic bacteria, were selected for by varying concentrations of NaCl. Scott (1957) reported that the nonhalophilic bacterium, S. aureus, was not selective in its response to NaCl concentrations ranging from 2.2 M to the absence of NaCl. Doudoroff (1940) demonstrated a significant increase in colony counts of E. coli when grown in gradually increasing NaCl concentrations up to 1.2 M compared to counts obtained when stressed with a sudden increase in 1.2 M NaCl. However, since adapted bacteria rapidly lost their increased ability to reproduce in saline media when transferred to a salt-free environment, Doudoroff interpreted the results to indicate that acclimatization was readily reversible. He concluded that the processes involved in adaptation could be separated into two components: (1) acclimatization, not involving reproduction (phenotypic adaptation), and (2) selection of the individuals with the greatest potentialities. The morphology of E. coli was altered (swollen cells) by the presence of NaCl, with normal morphology returning in a salt-free environment. Hof (1935) was unable to train E. coli to grow at 1.0 M NaCl. Thus, all attempts to alter the constancy of the NaCl response of nonhalophilic and obligate halophilic bacteria, including P. halodurans, were unsuccessful.

Oxygen utilization by P. halodurans was maximal at 0.20 - 0.30 M NaCl and decreased steadily at increased ionic strength in supplemental NaCl concentrations ranging from 1.30 to 4.30 M (Fig. 17). At 3.45 M supplemental NaCl, oxygen

utilization decreased nearly 80 % compared to maximal values (625 µliterO_2/mg dry weight). Increased NaCl stress has been observed to decrease respiration in a variety of terrestrial, marine, and moderate halophilic bacteria (Ingram, 1947). He attributed the inverse relation between increased NaCl and decreased respiration, in part, to the phenomenon of salting-out. This process influenced the solubility of dissolved proteins. However, Ingram did not determine whether the effect of NaCl on protein solubility was due to the combined ionic strength of both ions or to Na alone. Also, he did not explain adequately the effect of NaCl on halophilic bacterial respiration and concluded that the salting-out action only occurred with high molecular weight proteins. It appeared that the salting-out phenomenon was a localized event, occurring intracellularly in microenvironments of high NaCl concentrations. Additionally, the high NaCl concentrations caused conformational changes in proteins in a chain of respiratory enzymes (Brown, 1964). Yamada and Shiio (1953) demonstrated an inhibitory effect by NaCl concentrations greater than 1.5 M on the respiration of Bacillus pumilus var., a halotolerant bacterium. These investigators noted a supplemental inhibitory effect on respiration when 0.2 - 0.8 M glucose was added. They concluded that oxygen utilization was affected mainly by osmotic pressure, regardless of whether an electrolyte or nonelectrolyte was responsible for the osmotic pressure. Flannery et al. (1953) demonstrated an inverse relation between internal osmotic pressure and oxygen utilization in V. costicolus.

Eigen and Wilkens (1965) found an inverse relation between increased osmotic pressure (caused by 0.3 - 0.8 M NaCl) and decreased oxygen utilization by E. coli. They concluded that supplemental NaCl concentrations caused cell membrane distortion that affected membrane-bound respiratory enzymes as well as altering solute and water movement across the membrane. Contrary to the data of Yamada and Shiio (1953) and in light of the data that glycerol and sucrose did not alter the growth response of P. halodurans, oxygen utilization by the organism was affected only by an increase in the ionic strength of the medium.

Oxygen utilization by P. halodurans was decreased at NaCl concentrations below those of the estuary (Fig. 17). At 0.01 M NaCl, oxygen utilization decreased over 75 % compared to maximal values. At least 0.01 M NaCl was required for oxygen utilization by A. marinopraesens. The concentration of Na required was a function of the substrate oxidized (MacLeod et al., 1958). When the cell-free enzymes of the tricarboxylic acid cycle were studied, none were found to require Na for activity. Oddly, at Na concentrations required for maximal oxygen utilization, cell-free enzymes were inhibited. Pratt and Happold (1960) obtained similar results to those of MacLeod with a marine Vibrio. MacLeod et al. (1958) concluded that Na had specific functions in marine bacteria in transporting substrates into the cells and less specific functions in activating oxidative enzymes.

Na activated and combined with a permease or carrier molecule creating conformational changes in the molecules necessary for substrate affinity (Thompson and MacLeod, 1971). Low concentrations of Na activated less carrier molecules, less substrate uptake, and less oxygen utilization. Low concentrations of Na caused weakened cell wall and cell membrane, leading to disruption of membrane-bound respiratory enzymes as well as altering cell wall and cell membrane permeability which allowed oxidizable compounds to leave the cell (Tomlinson and MacLeod, 1957; Drapeau et al., 1966). It may be that at low concentrations of Na, K must assume a role of maintaining cell wall integrity. If so, then the amount of available K in the microenvironment surrounding the permease enzymes may be lower than needed for the optimal induction of a penetration and transport mechanism (Rhodes and Payne, 1962; Payne, 1960), and accumulation of exogeneous substrate within the cell membrane (Thompson and MacLeod, 1973). Osmotic imbalance may be a factor also in decreasing enzymatic activity of the penetration and transport system. No evidence has been found that a Na-pump was involved in the decrease in respiration at suboptimal NaCl concentrations (Drapeau et al., 1966). The exact reason for the reduced respiratory activity of P. halodurans at suboptimal NaCl concentrations is not known; however, any or all of the above-mentioned reasons for reduced respiration in other marine bacteria are probably applicable to P. halodurans.

Evidence that dehydrogenase enzyme(s) were inhibited at NaCl concentrations that deviated from those of the estuary occurred with the oxidation-reduction dyes, triphenyltetrazolium chloride (TTC), dichloroindophenol (DCIP), and methylene blue (MB) (Figs. 20-22). Dehydrogenase activity at 0.01 and 3.75 M NaCl decreased approximately 70 and 85 %, respectively, compared to maximal activity.

The exact reasons for the reduced enzyme activity in the electron transport system of P. halodurans are not known. A possible mechanism would be to explain the reduced activity in terms of elevated osmotic pressures caused by high NaCl concentrations which might alter one sensitive site or induce a whole sequence of secondary changes which may effect the physiological state of the whole cell (Passow et al., 1961). Alternate explanations may be possible permeability changes in the cell membrane to the oxidation-reduction compounds, a shift to an alternate electron transport pathway (Dolin, 1961), or ion antagonism by elevated internal NaCl concentrations rendering a flavoprotein nonfunctional (Rajagopalan and Handler, 1968). The similarity between the effect of supplemental NaCl on the respiration of P. halodurans and the amount and extent of reduction of TTC, DCIP, and MB, indicated that the reduced dehydrogenase activity at the flavoprotein-quinone level was due most probably to an elevated intracellular osmotic pressure created by the increased ionic strength of the medium. Reduced enzymatic activity at the flavoprotein-quinone level in the electron transport system

explained in part the decrease in the growth rate of P. halodurans as NaCl concentration increased.

The activity of the dehydrogenase enzyme(s) at 3.75 M NaCl indicated that the enzyme(s) were halotolerant. Yamada and Asano (1954) demonstrated a similar halotolerance of certain dehydrogenase enzymes isolated from Pseudomonas sp. No. 101. These enzymes tolerated up to 2.0 M NaCl with a 50 % decrease in activity compared to maximal activity at 0.3 M NaCl. The ability of dehydrogenase enzymes to function at 3.75 M explains in part the ability of P. halodurans to growth at, and tolerate, NaCl concentrations greater than 12-fold those of the estuary.

Supportive evidence that the tolerance of, and response to, supplemental NaCl by P. halodurans was reflective of the halotolerance of certain enzymes was found with the regulatory enzyme, aspartate transcarbamylase (ATCase). This allosteric enzyme involved in pyrimidine biosynthesis (Lehninger, 1975), demonstrated a halotolerant response to NaCl (up to 3.75 M) very similar to the response of whole cells (Fig. 31). Maximal enzyme activity (210 - 250 μM carbamyl aspartate/min/mg dry wt) occurred at NaCl concentrations approximating and slightly higher than those of the estuary (0.2 - 0.5 M) with activity decreasing steadily to a minimum of less than 15 % maximal at 3.75 M NaCl regardless of the growth medium NaCl concentration (Fig. 31). A partial explanation for the requirement for, and tolerance to, NaCl by P. halodurans was the requirement for NaCl by at least one regulatory enzyme of P. halodurans.

Liebl et al. (1969) demonstrated the dependence upon at least 2.0 M NaCl for the activity of ATCase extracted from H. cutirubrum. Baxter and Gibbons (1954, 1956, 1957) found such NaCl dependence was characteristic of a number of enzymes extracted from moderate and extreme halophilic bacteria. These investigators showed also that enzymes extracted from E. coli demonstrated maximal activity in the absence of NaCl. MacLeod et al. (1960) demonstrated an obligate NaCl requirement of 0.05 M NaCl for minimal activity of the aconitase and isocitrate dehydrogenases extracted from A. marinopraesens. Thus, similar to P. halodurans, the response of halophilic and nonhalophilic bacteria to NaCl reflected in part the response of their specific enzymes to NaCl. For P. halodurans, the decrease in ATCase activity at supplemental NaCl concentrations was reflected in the decrease in the growth rate. Thus, although the halotolerance of the enzyme permitted growth at increased NaCl concentrations, the reduction of enzymatic activity at these NaCl concentrations coincided with the reduced growth rate of P. halodurans.

The correlation between the growth rate of, and the halophilic character of enzymes isolated from, moderate and extreme halophilic bacteria, suggested that, contrary to theories that held that halophilic bacteria tolerated high concentrations of Na by excluding it actively from the cell (Robinson et al, 1952), intracellular Na approximated medium Na concentrations. The uptake and accumulation of Na and K by moderate and extreme halophilic bacteria was shown

subsequently by Christian (1956) as an integral part of halophilism.

The correlation between the response of cell-free ATCase and whole P. halodurans cells to supplemental NaCl to 3.75 M, indicated that the cells were not excluding ions from entering the cytoplasm and that the intracellular ionic concentrations of the cells was sufficiently high to affect enzymatic activity. Uptake and accumulation of Na and K by P. halodurans cells were dependent upon the physiological age of the culture and the concentration of NaCl in the medium (Tables 25 - 28). Maximum accumulation of Na and K occurred in the early logarithmic growth phase. At 345 mM medium Na, the intracellular Na averaged 423 mM which was 1.22 times the medium Na. The average intracellular K concentration at 12 mM medium K was 23 mM which was 1.92 times the medium K. The intracellular Na and K concentrations for P. halodurans in modified 2216E medium (345 mM NaCl) were similar to those obtained by Takacs et al. (1964) studying A. marinopraesens. With cells grown in medium NaCl concentrations ranging from 55 to 1020 mM, and K concentrations from 12 - 25 mM, these investigators found that the intracellular Na concentration was from 1.05 to 1.24 times greater than the medium Na concentration; while the intracellular K concentration was from 1.95 to 2.02 times greater than the medium K concentration. Thus, similar to A. marinopraesens, P. halodurans did not exclude Na or K from the interior of the cell.

In 1659 and 2920 mM medium Na, the intracellular Na concentration averaged 996 and 1198 mM, which was, respectively, 0.60 and 0.41 times the medium Na (Tables 26 and 27). At the same medium Na concentrations and 12 mM K, intracellular K averaged 169 and 306 mM, which was, respectively, 14.1 and 25.5 times the medium K. Masui and Wada (1973) using an unidentified moderate halophilic bacterium, No. 101, observed the intracellular K concentration varied from 121 to 161-fold the medium K (0.05M), as medium Na increased from 1.0 to 3.0 M. Intracellular Na concentration varied from 0.90 to 0.35 times the medium Na. Matheson et al. (1976) found that the intracellular Na and K concentrations of an unidentified moderate halophilic bacterium (NRCC No. 41227) varied with the age of the cells. The intracellular Na concentration never exceeded 0.25 fold the medium Na (0.50-4.52 M) while the intracellular K concentration varied between 10 and 15-fold the medium K (0.04 M). Christian and Waltho (1961) using stationary phase M. halodenitrificans and V. costicolus cells, grown in 1.0 M NaCl, found intracellular Na varied between 0.3 and 0.7-fold the medium Na while intracellular K varied between 55 and 120 fold-the medium K (0.004 M). These investigators studying H. salinarium and Halococcus morrhuae grown in 4.0 M NaCl and 0.032 M KCl to the stationary phase, noted intracellular Na concentrations varied between 0.5 and 0.8-fold that of the growth medium, while intracellular K was concentrated 64-140-fold the medium K. Although the intracellular concentrations of Na and K can vary a great

deal in moderate and extreme halophilic bacteria depending upon the age of the culture (Ginzburg et al., 1970; Schultz and Solomon, 1961) and the extent that Na and K exist in the free or bound state in the cell (Ginzburg et al., 1971; Lanyi and Silverman, 1972), the intracellular Na concentration, similar to NaCl-stressed P. halodurans cells, was never actively excluded from the cells and was dependent upon the medium Na concentration. Similar to moderate and extreme halophilic bacteria, the intracellular Na concentration of P. halodurans affected enzymatic activity. Thus, the response and tolerance of P. halodurans to supplemental NaCl concentrations up to 12-fold greater than those of the estuary must be partly attributed to the response and tolerance of ATCase to intracellular Na. The ratios, however, of DNA to RNA, protein, carbohydrate, and phospholipid were not altered by supplemental NaCl to 3.45 M (Tables 31 - 34). Thus, the enzymatic machinery for the production of five major biochemical constituents was not affected by the increased intracellular Na concentration. The constancy of the C:P and N:P ratios of P. halodurans during the extended lag phase of NaCl-stressed cells compared to unstressed cells indicated further, no ion-induced metabolic imbalance in the cells (Fig. 26). More enzymatic studies must be conducted before a thorough explanation of P. halodurans halotolerance can be advanced.

The accumulation of intracellular K in direct relation to NaCl concentration in the medium by P. halodurans

suggested that K may also contribute to the halotolerance of the organism. Christian and Waltho (1962) studying 38 species of gram-negative and gram-positive nonhalophilic and halophilic bacteria found that all species accumulated K in the cells between 10 and 140-fold the medium K as medium NaCl concentration increased. They noted a direct correlation between K content of the cell and NaCl requirement and tolerance of the species; the higher the degree of NaCl tolerance or requirement, the greater the accumulation of intracellular K. These results suggested two possibilities: (1) the K played an important role in the activation of the metabolic apparatus in halophilic bacteria; and/or (2) the high intracellular K imparted a resistance to plasmolysis and dehydration by lowering the water activity of halophilic and nonhalophilic NaCl tolerant cells, thus overcoming the detrimental effects of increased medium NaCl concentrations. The accumulation of intracellular K caused the Na/K ratio of the cytoplasm to decrease considerably when compared to the Na/K ratio of the medium. The Na/K of the growth medium used by Christian and Waltho (1962) was 6, 250, and 125, while the intracellular Na/K ratio averaged 0.3, 1.6, and 0.9, for nonhalophilic, moderate halophilic, and extreme halophilic bacteria, respectively. For P. halodurans the medium Na/K was approximately 30, 140, and 245, while the intracellular Na/K was approximately 18, 6, and 4, for the organism grown in modified 2216E medium and the medium supplemented with 1.30 and 2.60 M NaCl, respectively (Tables 25, 26, 27).

Thus, similar to halotolerant, nonhalophilic bacteria, and moderate and extreme halophilic bacteria, P. halodurans cells, contrary to Na accumulation, took up K actively from the medium. This active uptake of K by NaCl-stressed P. halodurans cells suggested strongly that K accumulation was involved in the halotolerance of the organism, although the exact basis of the relationship between intracellular K content and NaCl tolerance is not clear.

Concurrent with Na and K accumulation by P. halodurans cells in supplemented NaCl medium was an increase in the intracellular fluid volume (Tables 25, 26, 27). In 1.30 and 2.60 M supplemental NaCl-medium, intracellular fluid volume increased 2.5 and 4.5-fold, respectively, compared to the intracellular fluid volume of unstressed cells (approximately 2.0 ml/g dry weight). Takacs et al. (1964) found that the intracellular fluid volume of A. marinopraesens increased approximately two-fold (from 1.43 to 2.78 ml/g dry weight) as the medium NaCl concentration increased from 0.30 to 1.10 M. Yamada and Shiio (1953) found that the water content of the halotolerant organism, B. pumilus var., was a function of the intracellular Na and medium NaCl concentrations.

The correlation between increased intracellular fluid volume and increased concentrations of intracellular Na and K suggested that water entered P. halodurans cells passively to dilute and thereby reduce the potentially inhibitory effects that an increase in the intracellular ionic strength could cause on protein and enzyme activity. An increase in

the intracellular fluid caused an increase in cell size (volume) which could cause the cell membrane to press against the inner layer of the cell wall with a turgor pressure. Mitchell and Moyle (1956) observed the cell membrane to press against the cell wall of E. coli in 0.8 M NaCl. During balanced growth, the cell membrane was separated from the cell wall by the periplasmic space; and both membrane and wall expanded and changed shape (Salton, 1967). The maximum size a cell may attain was determined by the periplasmic space within the cell wall (Salton, 1967). If so, the maximum NaCl tolerance of P. halodurans may be in part a function of the expansion ability of the cell membrane. The distorted cell membrane could have caused the flagellum, already weakened by the increased intracellular and medium NaCl concentrations, to separate from the membrane-bound flagellar basal discs, rendering the cell nonmotile (Fig. 8). Once the NaCl stress was removed and the internal osmotic pressure decreased, the cell membrane returned to normal and the membrane associated-basal discs regenerated a new flagellum. Thus, the cells may not only have returned to the unstressed morphology, but also to the motile form. Strongly plasmolyzed Bacilli sp. were shown to first lose motility and then regain it when the cells were placed in a hypertonic and then isotonic medium (Doetsch and Cook, 1973; Vaituzis and Doetsch, 1969). Vaituzis and Doetsch (1969) concluded that flagellar basal discs were still intact and capable of regenerating flagella in isotonic solutions. Utilizing E. coli spheroplasts,

Vaituzis and Doetsch (1966) concluded that flagella synthesis involved a mechanism associated closely with the cytoplasmic membrane which was inactivated when the membrane was distorted by an increase in the ionic strength of the medium caused by supplemental NaCl.

Using ^{22}NaCl, P. halodurans accumulated from 14 to 22 % of the total cell-associated ^{22}Na at the cell envelope in modified 2216E medium and the medium supplemented with 1.30 and 2.60 M NaCl (Tables 29 and 30). D'Aoust and Kushner (1971) demonstrated the involvement of Na in membrane structure and cell wall stability of an unidentified psychrophilic marine bacterium. Costerton et al. (1967) indicated a possible role for Na in the cell wall stability of A. marinopraesens. Buckmire and MacLeod (1965), and Forsberg et al. (1970a) suggested that Na acted as a screen for negative charges permitting stabilization of the peptidoglycan layer. Devoe et al. (1970) found murein-dependent stability of the cell wall of a marine bacterium, C-A1, grown in 0.1 to 0.6 M NaCl. The ionic (especially Na) requirements of moderate and extreme halophilic bacteria have been explained by a similar mechanism (Brown, 1963; Larsen, 1962). Forsberg et al. (1970a) demonstrated the importance of the peptidoglycan layer for cell shape and wall integrity in A. marinopraesens grown in 0.05 to 0.8 M NaCl.

The accumulation of peptidoglycan-associated Na could be detrimental to the cell. P. halodurans cells released greater amounts of ultraviolet (UV)-absorbing material when

stressed with 1.70 and 3.45 M supplemental NaCl than unstressed cells (Fig. 27). Greater release of material occurred in the stationary phase than in the early logarithmic phase. The release of material may be caused by partial cytolysis of the cells, secretion, and/or alteration in the permeability of the cell membrane and cell wall.

Buckmire and MacLeod (1965, 1971) found that the permeability and stability of the membrane and envelope of A. marinopraesens were related to the availability of Mg to the cell. Recent findings have indicated the interaction of Mg with the peptidoglycan layer of the cell envelope of A. marinopraesens to involve the pentaglycine cross linkages (Rayman and MacLeod, 1975).

D'Aoust and Kushner (1971) demonstrated the involvement of Mg in membrane structure and wall stability of a psychrophilic marine bacterium. Devoe and Oginsky (1969a, b) noted the antagonistic effect of monovalent cations in maintaining cellular integrity in a marine isolate, C-A1, and A. marinopraesens. They determined that Na and Mg competed for the same electrostatic sites on the cell envelope with Na displacing Mg. They proposed that Mg held negatively charged subunits of the envelope together by divalent ionic bridges. Pretreatment with as little as 0.5 M NaCl resulted in exchange of Na for Mg and in the disappearance of the Mg bridges. The monovalent nature of Na caused a decrease in the electrostatic interactions with components such as anionic groups found in phosphoryl head groups of phospholipids or carboxyl groups

of protein side chains. This decrease in interactions led to a weakened cell envelope with subsequent release of UV-absorbing material (Forsberg et al., 1970a). A similar role for Mg has been postulated for EDTA-induced cytolysis of P. aeruginosa (Asbell and Eagon, 1966; Eagon, 1969). Soo-Hoo and Brown (1967) suggested bridging by Mg in the envlopes of halobacteria sp. as a mechanism to increase cell envelope integrity. In P. halodurans, the presence of supplemental Na replacing Mg in the cell envelope may increase permeability to H_2O while allowing UV-absorbing material to escape. The increase in UV-absorbing material as medium NaCl concentration increased, may also help explain the reduced growth of P. halodurans at increased ionic concentrations of modified 2216E medium.

Although supplemental NaCl altered the permeability of the cell membrane and/or cell wall of P. halodurans, NaCl concentrations of 2.0 and 3.75 M did not alter the qualitative and quantitative chemical composition of the cell envelope. The predominant chemical constituents on a dry weight basis were protein (73 %), carbohydrate (21 %), and total lipid (6 %) regardless of the medium NaCl concentration (Table 35). Reducing substance and hexosamine were both less than 2 %. The most abundant amino acids were aspartic acid, alanine, glycine, and leucine (Table 36). The most abundant phospholipid was phosphatidyl-ethanolamine (Fig. 28). Sud and Tyler (1964) examined three unidentified pseudomonad sp. and found protein, carbohydrate, and total lipid as the major cell wall

constituents with less than 2 % reducing substance and hexosamine. The qualitative and quantitative compositions of the cell envelopes of A. marinopraesens and two unidentified marine bacteria were similar to P. halodurans (Forsberg et al., 1972; Devoe and Oginsky, 1969b). The reduced hexosamine content resulted in a less rigid cell wall mucopeptide that could be a selective advantage to osmotically sensitive marine bacteria (Brown, 1960).

Transmission electron microscopy indicated no physical NaCl-induced weakening of the cell envelope of P. halodurans (Figs. 29 and 30). The appearance of the cell envelope resembled the typical gram-negative cell envelope of other marine bacteria (Costerton et al., 1974; Forsberg et al., 1972; Forsberg et al., 1970 a, b; Devoe and Oginsky, 1969a).

Brock (1969) suggested a number of ways in which an organism can adapt to or tolerate an environmental stress. One way was to live with the stress factor. This situation may in time produce an organism dependent upon the factor for its survival. P. halodurans tolerated supplemental NaCl, in part, by accumulating intracellular Na and K in proportion to medium NaCl concentrations. Thus, many enzymes may be exposed to the increased intracellular ionic strengths. However, if a high percentage of the intracellular Na and K existed in a bound, rather than free state, specific ionic-induced inhibition of enzymatic activity may play a small part in explaining reduced growth and growth rates at supplemental NaCl. However, both bound and free Na and K may contribute to an increase in

the internal osmotic pressure of P. halodurans which may inhibit enzymatic activity by inhibiting osmotically-sensitive enzymes.

P. halodurans is a marine bacterium unique in tolerance to supplemental NaCl greater than 12-fold that of the estuary. Why was the organism so halotolerant when the estuarine bacterial population as a whole was stenohaline to NaCl? The data from this dissertation indicated multiple answers. Firstly, the ability of specific respiratory enzymes, the dehydrogenases, to function at supplemental NaCl. Secondly, the halotolerance of at least one regulatory enzyme, ACTase, to 3.45 M NaCl. Thirdly, the active accumulation of K, although the exact relation between halotolerance and intracellular K content has remained obscure. Fourthly, the dilution of the intracellular elevated ionic concentration by intracellular fluid uptake was observed. Fifthly, the ability of the rigid peptidoglycan and flexible cell membrane prevented cellular bursting due to the increased internal osmotic pressure created by the uptake of water and intracellular Na and K. Finally, the ability of the enzymatic machinery involved in the biosynthesis of the major biochemical constituents as well as the chemical composition and physical integrity of the cell envelope resisted alteration by the increase in the intracellular ionic strength and osmotic pressure of the cytoplasm of P. halodurans. The tolerance of P. halodurans to NaCl may be due to one or more of the above-mentioned reasons. More studies on this and other halotolerant

bacteria must be conducted before an understanding of the evolutionary forces that created obligate halophilic bacteria can be determined.

TABLE 1

CFU/ml and percent CFU/ml of highest bacterial populations which developed from the Great Bay estuarine complex at 20 C after 14 days incubation on modified MacLeod's Na-deficient agar medium containing total NaCl concentrations ranging from 0.0002 to 3.20 M on 12/24/73 and 8/28/74.

	12/24/73		8/28/74	
Total NaCl, M	CFU/ml	% of Highest CFU/ml	CFU/ml	% of Highest CFU/ml
0.0002	833	22	867	22
0.05	933	24	933	23
0.10	2717	70	2633	66
0.20	3720	96	3733	93
0.30	3720	96	4010	100
0.40	3867	100	3667	91
0.50	3567	92	3520	88
1.00	1967	51	2067	52
1.40	233	6	167	4
1.65	33	1	37	1
1.90	20	1	23	1
2.30	17	0	13	0
2.80	3	0	3	0
3.20	0	0	0	0
Water Sample:				
Salinity, ppt	20.0		28.5	
Temperature, C	2.5		21.5	
pH	7.2		7.7	

TABLE 2

Percent CFU/ml of bacteria which developed from the Great Bay estuarine complex at 20 C after 14 days incubation on modified 2216E agar medium with supplemental NaCl concentrations ranging from 0.00 to 3.05 M on different sampling dates.

	Sampling Date					
Supplemental NaCl, M	10/29/73	11/25/73	12/30/73	1/21/74	2/20/74	4/30/74
CFU/ml=100%	2767	2207	2440	2366	1827	2763
0.00	100	100	100	100	100	100
0.25	78	73	69	74	76	78
0.45	61	63	67	66	67	62
0.65	51	54	52	48	49	50
0.85	27	30	26	30	23	26
1.00	18	20	16	17	15	18
1.25	6	6	5	7	7	8
1.45	3	3	2	2	2	3
1.65	1	1	1	2	1	1
1.85	1	0	1	1	0	0
2.25	0	0	0	0	0	0
2.65	0	0	0	0	0	0
3.05	0	0	0	0	0	0
Water Sample:						
Salinity, ppt	27.0	27.0	26.0	24.0	22.0	19.5
Temperature, C	10.5	6.5	5.0	1.5	0.5	10.5
pH	7.6	7.3	7.4	7.0	7.5	7.7

Table 2 (Continued)

Supplemental NaCl, M	Sampling Date 5/29/74	6/6/74	7/19/74	8/10/74	10/12/74	11/30/74	Ave. % Survival
CFU/ml= 100%	2840	2925	2507	3333	2623	2127	2560
0.00	100	100	100	100	100	100	100
0.25	81	77	79	77	73	78	76
0.45	64	66	70	65	65	67	65
0.65	53	58	51	51	56	57	53
0.85	29	29	30	22	20	22	26
1.00	21	24	20	19	22	20	19
1.25	7	7	7	8	7	7	7
1.45	3	2	3	2	3	3	3
1.65	2	1	2	1	1	2	1
1.85	1	1	1	0	0	1	0.5
2.25	0	0	0	0	0	0	0
2.65	0	0	0	0	0	0	0
3.05	0	0	0	0	0	0	0

Water Sample:

Salinity, ppt	23.0	25.5	27.0	28.5	26.0	23.5
Temperature, C	11.0	17.5	22.0	20.0	10.5	9.0
pH	6.8	7.9	7.7	7.8	7.5	7.5

TABLE 3

Pigmented and nonpigmented CFU/ml and percent pigmented CFU/ml of bacteria which developed from the Great Bay estuarine complex at 20 C after 14 days incubation on modified 2216E agar medium with supplemented NaCl concentrations ranging from 0.00 to 3.05 M.[1]

Supplemental NaCl, M	CFU/ml	Pigmented CFU/ml	Nonpigmented CFU/ml	% of Pigmented/ Nonpigmented Colonies
0.00	2560	650	1910	34
0.25	1907	439	1468	30
0.45	1754	351	1403	25
0.65	1408	260	1148	23
0.85	845	85	760	11
1.00	614	21	593	4
1.25	146	4	142	3
1.45	69	1	68	1
1.65	25	0	25	0
1.85	18	0	18	0
2.25	7	0	7	0
2.65	4	0	4	0
3.05	0	0	0	0

[1]Data based upon the mean of plates enumerated in Table 2.

TABLE 4

Percent CFU/ml of bacteria which developed from the Great Bay estuarine complex at 20 C after 14 days incubation on modified 2216E agar medium with supplemental salt concentrations ranging from 0.00 to 3.25 M on 3/12/74.

	Salt						
Supplemental Salt, M	$NaNO_3$	Na_2SO_4	KNO_3	KCl	$MgSO_4$	$CaSO_4$	LiCl
0.00	100	100	100	100	100	100	100
0.30	72	84	69	72	70	74	69
0.50	59	72	50	51	47	48	42
0.80	32	60	33	43	30	28	29
1.00	14	50	15	30	18	18	14
1.40	2	36	3	19	10	9	1
1.80	1	22	0	10	5	4	0
2.10	0	18	0	3	3	2	0
2.50	0	8	0	1	1	1	0
2.75	0	1	0	0	0	0	0
3.25	0	0	0	0	0	0	0

Water Sample:

Salinity	21.5
Temperature, C	2.5
pH	7.5

CFU/ml as 100 % was 2733.

TABLE 5

Percent CFU/ml of bacteria which developed from the Great Bay estuarine complex at 20 C after 14 days incubation on modified 2216E agar medium with supplemental salt concentrations ranging from 0.00 to 3.25 M on 8/21/74.

	Salt						
Supplemental Salt, M	$NaNO_3$	Na_2SO_4	KNO_3	KCl	$MgSO_4$	$CaSO_4$	LiCl
0.00	100	100	100	100	100	100	100
0.30	68	80	61	71	72	69	55
0.50	55	64	41	44	46	48	42
0.80	34	48	29	29	31	30	24
1.00	13	43	12	16	20	19	11
1.40	3	31	3	9	11	10	3
1.80	0	21	1	8	9	8	1
2.10	0	15	0	2	4	3	0
2.50	0	8	0	1	1	2	0
2.75	0	1	0	0	0	0	0
3.25	0	0	0	0	0	0	0

Water Sample:

Salinity, ppt	29.5
Temperature, C	21.0
pH	7.7

CFU/ml as 100 % was 2467.

TABLE 6

Determination of molar percent guanine plus cytosine by buoyant density and thermal melting (Tm) of DNA extracted from cells of a halotolerant estuarine bacterium (C-1) isolated from the Great Bay estuarine complex grown at 20 C and 200 rpm in modified 2216E medium with supplemental NaCl concentrations ranging from 0.0 to 3.45 M.

Supplemental NaCl, M	Density, g cm^{-3}	% GC	Tm C	% GC
0.0	1.722 ± 0.002	63.27 ± 0.01	75.5 ± 0.5	63.90 ± 0.92
1.70	1.722 ± 0.003	63.27 ± 0.01	75.0 ± 0.6	62.90 ± 1.21
2.60	1.722 ± 0.005	63.27 ± 0.01	75.2 ± 0.6	63.11 ± 0.94
3.45	1.722 ± 0.004	63.27 ± 0.01	75.0 ± 0.4	62.80 ± 1.01

TABLE 7

DNA-DNA hybridization studies for halotolerant isolate (C-1), isolated from the Great Bay estuarine complex grown at 20 C and 200 rpm to late logarithmic phase in (A) modified 2216E medium and (B) modified 2216E medium supplemented with 2.60 M NaCl.

Source of Unlabelled DNA	Unlabelled DNA, g/ml	^{32}P-DNA Source, g/ml		DPM ($\times 10^5$) Retained On Filter		% Hybridization		% ^{32}P-DNA Retained On Filter in Absence of Unlabelled DNA
		(A)	(B)	(A)	(B)[1]	(A)	(B)	
A	1	20.4	40.8	3.5	3.1	100.0	88.5	0.2
	5	20.4	40.8	4.4	4.0	100.0	90.9	0.7
	10	20.4	40.8	5.3	4.9	100.0	92.5	0.6
	20	20.4	40.8	6.8	6.2	100.0	91.2	0.5
B	1	20.4	40.8	3.0	2.7	90.0	100.0	0.8
	5	20.4	40.8	4.1	3.6	87.8	100.0	0.5
	10	20.4	40.8	4.8	4.4	91.7	100.0	0.6
	20	20.4	40.8	5.9	5.2	88.1	100.0	0.5
Pseudomonas sp. B-16 (*A. marinopraesens*)	10	20.4	40.8	3.3	3.1	62.5	64.3	-
	20	20.4	40.8	4.6	3.8	67.8	65.1	-
B. subtilis Phage *41 C*	10	20.4	40.8	0.04	0.03	0.75	0.63	-
	20	20.4	40.8	0.07	0.06	1.00	1.01	-

[1]Values corrected for ^{32}P-DNA concentration differences.

TABLE 8

Optical density of P. halodurans grown at 20 C and 200 rpm for 48 h in modified 2216E medium as a function of salinity.

Salinity ppt	Time, h									
	3	6	9	12	15	18	21	24	36	48
0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7.0	0.00	0.00	0.00	0.02	0.05	0.26	0.38	0.73	0.89	0.85
14.0	0.01	0.03	0.11	0.47	0.85	1.33	1.29	1.26	1.19	0.97
21.0	0.03	0.29	0.48	1.40	1.57	1.54	1.50	1.33	1.21	1.09
28.0	0.04	0.35	0.51	1.52	1.65	1.63	1.58	1.41	1.32	1.15
35.0	0.03	0.18	0.27	0.75	1.06	1.23	1.14	1.00	0.92	0.85
105	0.01	0.13	0.22	0.63	0.98	1.05	1.06	1.02	1.00	0.96
175	0.00	0.02	0.11	0.20	0.53	0.61	0.70	0.76	0.72	0.60
245	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
350	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

TABLE 9

Optical density of P. halodurans grown at 20 C and 200 rpm for 96 h in glucose seawater medium from which NaCl was deleted from synthetic seawater and osmotic pressure adjusted with 1.097 M glycerol as required.

	Time, h			
NaCl, mM	24	48	72	96
0.0	0.00	0.00	0.00	0.00
10	0.00	0.03	0.05	0.04
25	0.18	0.27	0.38	0.35
50	0.39	0.52	0.55	0.48
75	0.65	0.77	0.71	0.62
100	1.53	1.50	1.43	1.21
200	1.58	1.53	1.38	1.13
300	1.65	1.49	1.41	0.99
400	1.65	1.53	1.45	1.00
500	1.62	1.51	1.39	0.92

TABLE 10

Visual turbidity of P. halodurans grown at 20 C and 200 rpm for 24 h in glucose seawater medium from which specific salt concentrations were deleted from synthetic seawater and osmotic pressure adjusted with 1.097 M glycerol as required.

Treatment, #	Salt Deletion From Seawater	mM of Ions in Glucose Seawater Medium	Turbidity
1	No Na salts	0.00 Na	-
2	NaCl	43	-
3	NaF, $NaHCO_3$, Na_2SO_4	310	+++
4	NaF, $NaHCO_3$, 279 mM NaCl, 19 mM Na_2SO_4	35	+
5	NaF, $NaHCO_3$, 249 mM NaCl, 17 mM Na_2SO_4	69	++
6	NaF, $NaHCO_3$, 218 mM NaCl, 14.5 mM Na_2SO_4	104	+++
7	NaF, $NaHCO_3$, 187 mM NaCl, 13 mM Na_2SO_4	138	+++
8	Na_2SO_4	312	+++
9	$MgCl_2$	0.00 Mg	-
10	35 mM $MgCl_2$	4.0	-
11	31 mM $MgCl_2$	8.0	+
12	23.5 mM $MgCl_2$	16	++
13	$CaCl_2$	0.00 Ca	-
14	6.0 mM $CaCl_2$	1.5	-
15	4.0 mM $CaCl_2$	3.0	+

(Continued)

Table 10 (Continued)

Treatment, #	Salt Deletion From Seawater	mM of Ions in Glucose Seawater Medium	Turbidity
16	KCl	0.60 K	-
17	$SrCl_2$	0.00 Sr	+++
18	H_3BO_3	0.00 H_3BO_3	+++
19	Na_2SO_4, $NaHCO_3$, NaF, $SrCl_2$, H_3BO_3, KBr	7.0 K, 39 Mg 7.0 Ca, 310 Na	+++
20	Kcl, KBr, $SrCl_2$, H_3BO_3	39 Mg, 7.0 Ca, 35 Na	-

TABLE 11

Optical density of P. halodurans grown at 20 C and 200 rpm for 72 h in glucose seawater medium from which NaCl and KCl were deleted from synthetic seawater and osmotic pressure adjusted with 1.097 M glycerol as required. NaCl was increased from 0.0 to 500 mM as KCl was increased from 0.0 to 100 mM.

	Time, h								
	24			48			72		
	KCl, mM								
NaCl, mM	0.0	10	100	0.0	10	100	0.0	10	100
0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00	0.04	0.06	0.00	0.03	0.04
25	0.00	0.15	0.14	0.00	0.23	0.25	0.00	0.31	0.32
50	0.00	0.42	0.43	0.00	0.55	0.53	0.00	0.59	0.58
100	0.00	1.51	1.50	0.00	1.52	1.55	0.00	1.38	1.35
200	0.00	1.55	1.50	0.00	1.50	1.58	0.00	1.35	1.36
300	0.00	1.65	1.63	0.00	1.52	1.54	0.00	1.40	1.40
400	0.00	1.63	1.65	0.00	1.54	1.52	0.00	1.35	1.37
500	0.00	1.60	1.58	0.00	1.49	1.51	0.00	1.37	1.33

TABLE 12

Optical density of P. halodurans grown at 20 C and 200 rpm for 72 h in glucose seawater medium from which NaCl and KCl were deleted from synthetic seawater and osmotic pressure adjusted with 1.097 M glycerol as required. KCl was increased from 0.0 to 100 mM as NaCl was increased from 0.0 to 500 mM.

	Time, h											
	24				48				72			
	NaCl, mM											
KCl, mM	0.0	50	200	500	0.0	50	200	500	0.0	50	200	500
0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	0.03	0.05	0.00	0.00	0.10	0.18	0.00	0.00	0.05	0.13
4	0.00	0.00	0.05	0.08	0.00	0.07	0.37	0.35	0.00	0.03	0.29	0.26
6	0.00	0.09	0.73	0.77	0.00	0.21	0.98	1.03	0.00	0.25	0.96	0.97
8	0.00	0.28	1.45	1.46	0.00	0.36	1.40	1.46	0.00	0.38	1.23	1.31
10	0.00	0.53	1.58	1.60	0.00	0.67	1.53	1.58	0.00	0.65	1.42	1.40
50	0.00	0.65	1.55	1.56	0.00	0.73	1.51	1.49	0.00	0.75	1.44	1.45
90	0.00	0.59	1.57	1.53	0.00	0.64	1.50	1.51	0.00	0.69	1.39	1.37
100	0.00	0.61	1.54	1.54	0.00	0.68	1.52	1.48	0.00	0.73	1.40	1.36

TABLE 13

Optical density of P. halodurans grown at 20 C and 200 rpm for 72 h in glucose seawater medium from which $MgCl_2$ and $CaCl_2$ were deleted from synthetic seawater and osmotic pressure adjusted with 1.097 M glycerol as required. $MgCl_2$ was increased from 0.0 to 100 mM as $CaCl_2$ was increased from 0.0 to 100 mM.

	Time, h								
	24			48			72		
	$CaCl_2$, mM								
$MgCl_2$, mM	0.0	10	100	0.0	10	100	0.0	10	100
0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	0.00	0.25	0.29	0.00	0.68	0.71	0.00	0.69	0.72
25	0.00	0.78	0.74	0.00	1.21	1.26	0.00	1.18	1.22
50	0.00	1.63	1.61	0.00	1.60	1.58	0.00	1.51	1.53
100	0.00	1.59	1.57	0.00	1.55	1.52	0.00	1.50	1.47

TABLE 14

Optical density of P. halodurans grown at 20 C and 200 rpm for 72 h in glucose seawater medium from which $MgCl_2$ and $CaCl_2$ were deleted from synthetic seawater and osmotic pressure adjusted with 1.097 M glycerol as required. $CaCl_2$ was increased from 0.0 to 50 mM as $MgCl_2$ was increased from 0.0 to 100 mM.

	Time, h								
	24			48			72		
	$MgCl_2$, mM								
$CaCl_2$, mM	0.0	50	100	0.0	50	100	0.0	50	100
0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.00	0.00	0.00	0.05	0.06	0.00	0.03	0.03
3	0.00	0.05	0.09	0.00	0.18	0.17	0.00	0.11	0.12
5	0.00	0.55	0.52	0.00	0.63	0.66	0.00	0.58	0.59
10	0.00	1.60	1.61	0.00	1.58	1.56	0.00	1.52	1.49
25	0.00	1.55	1.51	0.00	1.50	1.51	0.00	1.48	1.45
50	0.00	1.57	1.53	0.00	1.52	1.49	0.00	1.47	1.44

TABLE 15

Optical density of P. halodurans grown at 20 C and 200 rpm for 72 h in glucose seawater medium in which the anion concentrations were all Cl, Br, or SO_4, respectively.

	Time, h								
	Cl			Br			SO_4		
Anion Added, mM	24	48	72	24	48	72	24	48	72
0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.02	0.15	0.36	0.03	0.11	0.32	0.05	0.14	0.39
10	0.28	0.51	0.49	0.25	0.53	0.52	0.26	0.49	0.39
50	0.58	0.67	0.63	0.51	0.62	0.66	0.50	0.63	0.48
100	0.99	0.88	0.84	1.00	0.79	0.81	0.97	0.82	0.73
200	1.11	1.00	0.93	1.05	0.98	0.92	1.02	1.00	0.88

TABLE 16

CFU/ml, direct cell count/ml, optical density, and dry weight of P. halodurans grown at 20 C and 200 rpm for 72 h in modified 2216E medium with supplemental NaCl concentrations ranging from 0.0 to 2.60 M.

	Supplemental NaCl, M							
	0.0				0.90			
Time, h	CFU/ml, X 10^8	Direct Count/ml, X 10^8	Optical Density	Dry Weight, mg/ml	CFU/ml, X 10^8	Direct Count/ml, X 10^8	Optical Density	Dry Weight mg/ml
3	0.42	0.47	0.05	0.024	-	-	-	-
4.5	2.82	3.29	0.25	0.231	1.18	1.32	0.13	0.076
6	10.0	11.8	0.55	1.12	2.06	2.48	0.19	0.151
9	56.1	62.3	1.00	6.47	27.2	28.1	0.68	1.68
12	102.0	105.0	1.51	12.95	68.1	72.4	1.20	8.91
15	113.0	119.0	1.65	13.37	102.0	104.0	1.55	13.03
18	118.0	129.0	1.65	13.66	106.0	109.0	1.65	13.22

(Continued)

TABLE 16 (continued)

	Supplemental NaCl, M							
	1.70				2.60			
Time, h	CFU/ml, X 10^8	Direct Count/ml, X 10^8	Optical Density	Dry Weight, mg/ml	CFU/ml, X 10^8	Direct Count/ml, X 10^8	Optical Density	Dry Weight, mg/ml
10	0.89	1.00	0.14	0.100	-	-	-	-
12	2.22	2.48	0.23	0.376	0.24	0.31	0.04	0.060
16	9.02	9.62	0.50	1.92	0.69	0.78	0.16	0.153
20	38.9	42.3	0.79	7.73	2.11	2.51	0.28	0.527
24	51.3	52.7	1.05	9.06	5.84	6.25	0.42	1.63
36	59.5	61.6	1.20	10.48	16.9	17.7	0.63	4.21
48	62.2	63.0	1.18	11.65	20.4	21.0	0.87	7.24
72	-	-	-	-	20.7	21.6	0.91	8.00

TABLE 17

Specific growth rate, lag phase, and maximum optical density of P. halodurans grown at 20 C and 200 rpm in modified 2216E medium and glucose seawater medium both with supplemental NaCl concentrations ranging from 0.0 to 4.30 M.

	Modified 2216E Medium			Glucose Seawater Medium		
Supplemental NaCl, M	Specific Growth Rate, Generation/h	Lag Phase, h	Maximum Optical Density	Specific Growth Rate, Generation/h	Lag Phase, h	Maximum Optical Density
0.0	0.325 ± 0.04	3.0 ± 0.5	1.65 ± 0.05	0.321 ± 0.04	3.0 ± 0.5	1.65 ± 0.05
0.90	0.320 ± 0.04	4.5 ± 0.5	1.65 ± 0.06	0.319 ± 0.03	4.5 ± 0.5	1.65 ± 0.05
1.70	0.257 ± 0.02	9.5 ± 0.5	1.22 ± 0.03	0.259 ± 0.02	10 ± 1.0	1.18 ± 0.04
2.60	0.131 ± 0.03	16 ± 0.9	0.95 ± 0.04	0.128 ± 0.04	15 ± 1.5	0.94 ± 0.02
3.45	0.036 ± 0.003	58 ± 1.5	0.38 ± 0.03	0.033 ± 0.004	61 ± 2.0	0.37 ± 0.02
4.30	0	0	0	0	0	0

TABLE 18

Specific growth rate, lag phase , and maximum optical density of P. halodurans grown at 20 C and 200 rpm in modified 2216E medium with supplemental glycerol and sucrose concentrations ranging from 0.0 to 3.5 M.

	Glycerol			Sucrose		
Concentration, M	Specific Growth Rate, Generation/h	Lag Phase , h	Maximum Optical Density	Specific Growth Rate, Generation/h	Lag Phase , h	Maximum Optical Density
0.0	0.321 ± 0.03	3.0 ± 0.5	1.55 ± 0.05	0.322 ± 0.04	3.5 ± 0.5	1.60 ± 0.05
1.0	0.320 ± 0.04	3.0 ± 0.5	1.50 ± 0.04	0.325 ± 0.04	3.0 ± 0.5	1.55 ± 0.04
1.5	0.322 ± 0.02	3.5 ± 0.5	1.60 ± 0.06	0.321 ± 0.05	3.5 ± 0.5	1.60 ± 0.06
2.0	0.321 ± 0.02	3.5 ± 0.5	1.55 ± 0.05	0.318 ± 0.05	3.5 ± 0.5	1.55 ± 0.06
2.5	0.319 ± 0.05	3.0 ± 0.5	1.60 ± 0.03	0.318 ± 0.03	3.0 ± 0.5	1.60 ± 0.05
3.0	0.325 ± 0.04	3.0 ± 0.5	1.65 ± 0.05	0.322 ± 0.04	3.5 ± 0.5	1.65 ± 0.04
3.5	0.324 ± 0.05	3.0 ± 0.5	1.55 ± 0.06	0.324 ± 0.05	3.0 ± 0.5	1.60 ± 0.05

TABLE 19

Specific growth rate, lag phase , maximum optical density, and cell size of P. halodurans grown at 20 C and 200 rpm in modified 2216E medium with supplemental salt concentrations ranging from 0.0 to 3.45 M.

Salt	Supplemental Salt, M	Specific Growth Rate, Generation/h	Lag Phase , h	Maximum Optical Density	Cell Size, µm
$NaNO_3$	0.00	0.33	3.0	1.65	0.7 X 1.5
	0.90	0.32	4.0	1.30	0.7 X 1.5
	1.70	0.26	10.5	0.92	1.2 X 3.0
	2.60	0.11	19.5	0.61	1.5 X 3.0
	3.45	0	0	0	0
Na_2SO_4	0.00	0.33	3.0	1.65	0.7 X 1.5
	0.90	0.32	3.0	1.60	0.7 X 1.5
	1.70	0.28	5.5	1.25	1.0 X 2.0
	2.60	0.26	12.5	0.96	1.5 X 2.5
	3.45	0.03	24.0	0.23	2.0 X 4.0

(Continued)

Table 19 (Continued)

Salt	Supplemental Salt, M	Specific Growth Rate, Generation/h	Lag Phase, h	Maximum Optical Density	Cell Size, μm
KCl	0.00	0.33	3.0	1.65	0.7 X 1.5
	0.90	0.32	4.0	1.50	0.7 X 1.5
	1.70	0.30	5.0	1.30	2.0 X 3.0
	2.60	0.23	8.0	1.02	2.0 X 3.0
	3.45	0.19	16.5	0.86	2.0 X 3.0
KNO_3	0.00	0.33	3.0	1.65	0.7 X 1.5
	0.90	0.31	4.5	1.20	0.7 X 1.5
	1.70	0.13	6.0	0.95	1.0 X 2.0
	2.60	0.05	15.0	0.43	1.0 X 2.0
	3.45	0	0	0	0
LiCl	0.00	0.33	3.0	1.65	0.7 X 1.5
	0.90	0.15	5.5	0.81	0.7 X 1.5
	1.70	0.03	15.0	0.20	0.7 X 1.5

(Continued)

Table 19 (Continued)

Salt	Supplemental Salt, M	Specific Growth Rate, Generation/h	Lag Phase h	Maximum Optical Density	Cell Size, μm
LiCl (Cont.)	2.60	0	0	0	0
	3.45	0	0	0	0
NH_4Cl	0.00	0.33	3.0	1.65	0.7 X 1.5
	0.90	0.07	8.0	0.45	0.7 X 1.5
	1.70	0.02	34.5	0.13	0.7 X 1.5
	2.60	0	0	0	0
	3.45	0	0	0	0
$MgCl_2$	0.00	0.33	3.0	1.65	0.7 X 1.5
	0.90	0.32	4.0	1.55	0.7 X 1.5
	1.70	0.25	6.5	1.11	1.0 X 2.0
	2.60	0.08	17.5	0.53	1.0 X 2.0
	3.45	0	0	0	0

(Continued)

Table 19 (Continued)

Salt	Supplemental Salt, M	Specific Growth Rate, Generation/h	Lag Phase, h	Maximum Optical Density	Cell Size, μm
$CaCl_2$	0.00	0.33	3.0	1.65	0.7 X 1.5
	0.90	0.32	4.0	1.53	0.7 X 1.5
	1.70	0.05	17.0	0.48	1.0 X 2.0
	2.60	0.02	33.0	0.11	1.0 X 2.0
	3.45	0	0	0	0

TABLE 20

Specific growth rate, lag phase, and maximum optical density of P. halodurans grown at 20 C and 200 rpm in modified 2216E medium and supplemented with 3.45 M NaCl and subsequently grown in modified 2216E medium with supplemental NaCl concentrations ranging from 0.0 to 4.30 M.

	0.30 M NaCl			3.75 M NaCl		
Supplemental NaCl, M	Specific Growth Rate, Generation/h	Lag Phase, h	Maximum Optical Density	Specific Growth Rate, Generation/h	Lag Phase h	Maximum Optical Density
0.0	0.324 ± 0.03	3.0 ± 0.5	1.65 ± 0.04	0.326 ± 0.04	3.5 ± 0.5	1.60 ± 0.05
0.90	0.319 ± 0.03	4.5 ± 0.6	1.65 ± 0.06	0.317 ± 0.03	4.5 ± 0.6	1.60 ± 0.03
1.70	0.256 ± 0.03	10 ± 1.0	1.20 ± 0.03	0.252 ± 0.02	11 ± 1.5	1.18 ± 0.04
2.60	0.133 ± 0.01	15 ± 0.8	0.93 ± 0.05	0.135 ± 0.01	16 ± 1.7	0.91 ± 0.06
3.45	0.032 ± 0.002	60 ± 1.3	0.39 ± 0.04	0.028 ± 0.002	63 ± 2.0	0.38 ± 0.05
4.30	0	0	0	0	0	0

TABLE 21

CFU/ml on initial plate, CFU/ml on replica plate, and colonies (% of 26 ppt medium) of *P. halodurans* grown at 20 C and 200 rpm in modified 2216E medium and supplemented with 3.45 M NaCl and subsequently grown at 20 C for 10 days on modified 2216E agar medium with total NaCl concentrations ranging from 0.01 to 5.40 M.

	0.30 M NaCl			3.75 M NaCl		
Total NaCl, M	CFU/ml on Initial Plate	CFU/ml on Replica Plate	Colonies, % of Medium	CFU/ml on Initial Plate	CFU/ml on Replica Plate	Colonies, % of Medium
0.01	36	36	32	34	33	30
0.05	87	86	78	83	83	76
0.10	96	95	87	95	95	87
0.20	101	101	91	100	99	91
0.25	109	106	98	103	101	93
0.30	111	110	100	109	109	100
1.20	98	96	88	99	96	88
2.00	89	86	80	87	86	79
2.90	77	75	70	69	69	63
3.75	24	23	22	20	20	18
4.60	0	0	0	0	0	0
5.40	0	0	0	0	0	0

TABLE 22

Specific growth rate, lag phase , maximum optical density, and cell size of *P. halodurans*, *A. marinus*, *P. cuprodurans*, *Pseudomonas* sp. 130, and *Alteromonas marinopraesens* grown at 20 C and 200 rpm in modified 2216E medium with supplemental NaCl concentrations ranging from 0.0 to 3.45 M.

Bacterial Culture	Supplemental NaCl, M	Specific Growth Rate, Generation/h	Lag Phase , h	Maximum Optical Density	Cell Size, μm
P. halodurans	0.00	0.32	3.0	1.65	0.5 X 1.5
	0.90	0.32	4.5	1.65	0.7 X 2.0
	1.70	0.26	10.5	1.20	1.0 X 2.3
	2.60	0.13	15.0	0.93	1.5 X 3.0
	3.45	0.03	60.0	0.39	1.5 X 4.0
A. marinus	0.00	0.37	3.0	0.87	0.8 X 1.5
	0.90	0.33	5.5	0.78	0.8 X 1.5
	1.70	0.18	15.0	0.61	1.0 X 2.0
	2.60	0.03	72	0.34	1.0 X 3.0
	3.45	0.0	0	0	0
P. cuprodurans	0.00	0.42	3.0	1.25	1.0 X 2.0
	0.90	0.41	4.0	1.18	1.0 X 2.0
	1.70	0.21	12.5	0.56	1.0 X 2.5
	2.60	0.03	80	0.19	1.0 X 3.5
	3.45	0.0	0	0	0
Pseudomonas sp. 130	0.00	0.35	3.5	1.10	1.0 X 2.0
	0.90	0.31	6.0	0.91	1.0 X 2.0
	1.70	0.10	67.0	0.23	1.5 X 2.0
	2.60	0.0	0	0	0
	3.45	0.0	0	0	0
Alteromonas marinopraesens	0.00	0.35	3.0	1.00	1.0 X 1.5
	0.90	0.05	70	0.39	1.0 X 1.5
	1.70	0.01	122.0	0.08	1.0 X 1.5
	2.60	0.0	0	0	0
	3.45	0.0	0	0	0

TABLE 23

Specific growth rate, lag phase , maximum optical density, and cell size of *P. halodurans* (C-1) and bacteria isolated from the Great Bay estuarine complex, grown at 20 C and 200 rpm in modified 2216E medium with supplemental NaCl concentrations ranging from 0.0 to 3.45 M.

Bacterial Culture	Supplemental NaCl, M	Specific Growth Rate, Generation/h	Lag Phase, h	Maximum Optical Density	Cell Size, μm
P. halodurans (C-1)	0.00	0.32	3.0	1.65	0.5 X 1.5
	0.90	0.32	4.5	1.65	0.7 X 2.0
	1.70	0.26	10.5	1.20	1.0 X 2.3
	2.60	0.13	15.0	0.93	1.5 X 3.0
	3.45	0.03	60.0	0.39	1.5 X 4.0
C-2	0.00	0.30	4.5	1.15	1.0 X 2.0
	0.90	0.16	17.0	0.93	1.0 X 2.5
	1.70	0	0	0	0
	2.60	0	0	0	0
	3.45	0	0	0	0
C-3	0.00	0.32	3.5	1.33	1.0 X 3.0
	0.90	0.32	4.0	1.30	1.0 X 3.0
	1.70	0.13	10.5	0.89	1.0 X 3.0
	2.60	0	0	0	0
	3.45	0	0	0	0
C-4	0.00	0.25	6.0	1.18	1.0 X 2.5
	0.90	0.10	11.5	0.85	1.0 X 3.0
	1.70	0.04	18.5	0.22	1.0 X 2.5
	2.60	0	0	0	0
	3.45	0	0	0	0
C-5	0.00	0.32	3.5	1.27	1.5 X 5.0
	0.90	0.32	4.0	1.19	1.5 X 5.5
	1.70	0.12	15.0	0.75	1.5 X 5.5
	2.60	0.04	26.5	0.32	1.5 X 5.0
	3.45	0	0	0	0

TABLE 24

Reduction time of triphenyl tetrazolium chloride by P. halodurans grown at 20 C on modified 2216E agar medium with total NaCl concentrations ranging from 0.01 to 3.75 M using 1.5 % agar or 10 % gelatin overlays.

Total NaCl, M	Time, min	
	1.5 % Agar Overlay	10 % Gelatin Overlay
0.01	35	38
0.10	7.5	8.0
0.20	5.0	5.0
0.25	5.0	5.0
0.30	5.0	6.0
1.20	7.0	7.0
1.60	8.5	9.0
2.00	10	10
2.90	32	35
3.75	55	57

TABLE 25

Intracellular concentrations of Na and K (mM) in cells of *P. halodurans* grown at 20 C and 200 rpm to early logarithmic (EL), mid-logarithmic (ML), late logarithmic (LL), and stationary (ST) phase of growth in modified 2216E medium.

Determination	Growth Phase EL	ML	LL	ST
g dry wt to g wet wt ratio	0.30 ± 0.05	0.29 ± 0.07	0.26 ± 0.06	0.29 ± 0.06
Na associated with cells[1]	827 ± 103	761 ± 31	711 ± 66	647 ± 51
^{22}Na ratio method				
Direct flame photometry	970 ± 118	811 ± 38	674 ± 51	598 ± 39
K associated with cells[1]	41.4 ± 1.5	40.9 ± 3.2	44.3 ± 1.9	39.9 ± 2.2
Intracellular fluid volume[2]	1.99 ± 0.10	1.87 ± 0.10	1.92 ± 0.06	1.91 ± 0.10
Intracellular Na concentration	483 ± 38	436 ± 36	406 ± 39	367 ± 38
Intracellular K concentration	25.1 ± 2.2	23.4 ± 1.8	23.2 ± 2.1	20.5 ± 2.7
Intracellular Na/medium Na	1.38 ± 0.13	1.28 ± 0.09	1.16 ± 0.10	1.05 ± 0.10
Intracellular K/medium K	2.09 ± 0.15	1.95 ± 0.11	1.93 ± 0.11	1.71 ± 0.15
Intracellular Na/intracellular K	19.2 ± 1.7	18.6 ± 1.8	17.5 ± 1.4	17.9 ± 1.4
Medium Na/medium K	29.2 ± 2.1	29.5 ± 2.1	30.2 ± 2.1	30.3 ± 2.1

[1]Expressed as μM/g dry weight.

[2]Expressed as ml/g dry weight.

TABLE 26

Intracellular concentrations of Na and K (mM) in cells of P. halodurans grown at 20 C and 200 rpm to early logarithmic (EL), mid-logarithmic (ML), late logarithmic (LL), and stationary (ST) phase of growth in modified 2216E medium supplemented with 1.30 M NaCl.

Determination	Growth Phase EL	ML	LL	ST
g dry wt to g wet wt ratio	0.38 ± 0.04	0.33 ± 0.09	0.32 ± 0.03	0.35 ± 0.05
Na associated with cells[1]				
^{22}Na ratio method	2529 ± 158	2052 ± 81	2011 ± 58	1611 ± 41
Direct flame photometry	2438 ± 141	2111 ± 96	1925 ± 49	1711 ± 38
K associated with cells[1]	322 ± 16	283 ± 10	270 ± 11	263 ± 12
Intracellular fluid volume[2]	5.27 ± 0.11	4.68 ± 0.11	5.05 ± 0.08	5.03 ± 0.06
Intracellular Na concentration	1178 ± 68	1045 ± 78	913 ± 55	846 ± 55
Intracellular K concentration	213 ± 42	182 ± 51	149 ± 22	131 ± 18
Intracellular Na/medium Na	0.71 ± 0.09	0.63 ± 0.08	0.55 ± 0.05	0.51 ± 0.06
Intracellular K/medium K	17.8 ± 1.1	15.2 ± 1.7	12.4 ± 2.3	10.9 ± 1.1
Intracellular Na/intracellular K	5.53 ± 0.08	5.74 ± 0.11	6.13 ± 0.06	6.46 ± 0.10
Medium Na/medium K	138 ± 9	140 ± 10	137 ± 8	138 ± 9

[1]Expressed as μ M/g dry weight.

[2]Expressed as ml/g dry weight.

TABLE 27

Intracellular concentrations of Na and K (mM) in cells of P. halodurans grown at 20 C and 200 rpm to early logarithmic (EL), mid-logarithmic (ML), late logarithmic (LL), and stationary (ST) phase of growth in modified 2216E medium supplemented with 2.60 M NaCl.

Determination	Growth Phase			
	EL	ML	LL	ST
g dry wt to g wet wt ratio	0.48 ± 0.03	0.44 ± 0.07	0.39 ± 0.02	0.43 ± 0.02
Na associated with cells[1]				
^{22}Na ratio method	3508 ± 210	2997 ± 111	2712 ± 103	2349 ± 125
Direct flame photometry	3221 ± 187	2439 ± 105	2502 ± 100	2441 ± 168
K associated with cells[1]	476 ± 27	450 ± 37	389 ± 12	369 ± 18
Intracellular fluid volume[2]	8.49 ± 0.08	7.92 ± 0.05	8.01 ± 0.12	7.93 ± 0.10
Intracellular Na concentration	1382 ± 52	1220 ± 89	1138 ± 78	1052 ± 61
Intracellular K concentration	385 ± 34	343 ± 31	261 ± 21	235 ± 25
Intracellular Na/medium Na	0.47 ± 0.03	0.42 ± 0.05	0.39 ± 0.05	0.36 ± 0.04
Intracellular K/medium K	32.1 ± 2.9	28.6 ± 2.2	21.8 ± 2.8	19.6 ± 1.8
Intracellular Na/intracellular K	3.60 ± 0.05	3.56 ± 0.05	4.36 ± 0.06	4.48 ± 0.06
Medium Na/medium K	246 ± 13	248 ± 13	244 ± 11	249 ± 11

[1]Expressed as μM/g dry weight.

[2]Expressed as ml/g dry weight.

TABLE 28

Intracellular concentrations of Na and K (mM) in cells of P. halodurans grown at 20 C and 200 rpm to early logarithmic phase (OD 420 nm = 0.25) in modified 2216E medium and subsequently stressed with 2.60 M NaCl for 24, 48, and 72 h.

Determination	Time, h, After 2.60 M NaCl Addition			
	0	24	48	72
g dry wt to g wet wt ratio	0.23 ± 0.03	0.50 ± 0.07	0.41 ± 0.05	0.40 ± 0.05
Na associated with cells[1]				
^{22}Na ratio method	822 ± 98	4458 ± 225	3591 ± 79	3199 ± 113
Direct flame photometry	875 ± 105	4639 ± 211	3638 ± 87	3303 ± 121
K associated with cells[1]	40.9 ± 1.1	410 ± 22	375 ± 16	371 ± 13
Intracellular fluid volume[2]	1.87 ± 0.09	4.21 ± 0.12	6.87 ± 0.09	8.53 ± 0.11
Intracellular Na concentration	458 ± 89	2146 ± 89	1522 ± 65	1021 ± 49
Intracellular K concentration	24.4 ± 1.7	288 ± 44	241 ± 37	228 ± 33
Intracellular Na/medium Na	1.31 ± 0.12	0.73 ± 0.08	0.52 ± 0.07	0.35 ± 0.06
Intracellular K/medium K	2.04 ± 0.14	24.0 ± 0.9	20.1 ± 1.1	19.0 ± 1.2
Intracellular Na/intracellular K	18.8 ± 1.5	7.45 ± 0.09	6.32 ± 0.07	4.48 ± 0.05
Medium Na/medium K	29.2 ± 2.0	245 ± 12	250 ± 13	248 ± 12

[1]Expressed as µM/g dry weight.

[2]Expressed as ml/g dry weight.

TABLE 29

Physical location and ^{22}Na associated with cells of P. halodurans grown at 20 C and 200 rpm to early logarithmic (EL), mid-logarithmic (ML), late logarithmic (LL), and stationary (ST) phase of growth in modified 2216E medium supplemented with 1.30 and 2.60 M NaCl. All values are 10^6 DPM.

	Supplemental NaCl, M											
	0.0				1.30				2.60			
Determination	EL	ML	LL	ST	EL	ML	LL	ST	EL	ML	LL	ST
Total DPM associated with cells	0.29	0.17	0.07	0.07	1.71	0.69	0.40	0.22	5.81	2.13	1.02	0.79
Total DPM added	6.66	6.66	6.66	6.66	33.0	33.0	33.0	33.0	66.0	66.0	66.0	66.0
% of total DPM added, associated with cells	4.35	2.55	1.05	1.05	5.1	2.09	1.21	0.66	8.80	3.23	1.54	1.20
Total DPM associated with cell envelopes	0.06	0.03	0.01	0.01	0.34	0.11	0.06	0.03	1.28	0.38	0.15	0.10
% of total DPM added, associated with cell envelopes	0.90	0.45	0.15	0.15	1.03	0.33	0.18	0.09	1.93	0.58	0.23	0.15
% of total DPM associated with cells, after association with cell envelopes	20.69	17.64	14.29	14.29	19.88	15.94	15.01	13.63	22.03	17.84	14.71	12.66

TABLE 30

Physical location and ^{22}Na associated with cells of P. halodurans grown at 20 C and 200 rpm to early logarithmic phase ($OD_{420\ nm}$=0.25) in modified 2216E medium and subsequently stressed with 2.60 M NaCl for 24, 48, and 72 h. All values are 10^6 DPM.

	Time, h, After 2.60 M NaCl Addition			
Determination	0	24	48	72
Total DPM associated with cells	0.30	4.93	2.82	2.51
Total DPM added	6.66	66.0	66.0	66.0
% of total DPM added, associated with cells	4.50	7.47	4.27	3.80
Total DPM associated with cell envelopes	0.06	0.84	0.46	0.38
% of total DPM added, associated with cell envelopes	0.90	1.27	0.70	0.58
% of total DPM associated with cells, after association with cell envelopes	20.00	17.02	16.14	14.99

TABLE 31

Total concentration and ratio of DNA:RNA:protein:carbohydrate:phospholipid for P. halodurans grown at 20 C and 200 rpm to the early logarithmic phase in modified 2216E medium with supplemental NaCl concentrations ranging from 0.0 to 3.45 M.

Supplemental	DNA		RNA		Protein		Carbohydrate		Phospholipid	
NaCl, M	µg/ml	DNA/DNA	µg/ml	RNA/DNA	µg/ml	Prot/DNA	µg/ml	Carb/DNA	µg/ml	Phos/DNA
0.0	35	1.00	106	3.03	413	11.80	300	8.58	10	0.29
0.0	30	1.00	93	3.08	357	11.91	253	8.43	8	0.27
0.90	31	1.00	95	3.05	358	11.52	267	8.61	8	0.26
0.90	29	1.00	89	3.08	335	11.56	249	8.59	9	0.30
1.70	26	1.00	79	3.04	301	11.59	221	8.51	7	0.26
1.70	22	1.00	66	3.01	255	11.57	188	8.55	6	0.27
2.60	11	1.00	34	3.11	128	11.61	95	8.60	3	0.31
2.60	10	1.00	31	3.06	117	11.71	85	8.53	3	0.25
3.45	6	1.00	18	3.02	70	11.63	51	8.49	2	0.26
3.45	6	1.00	18	3.03	71	11.68	50	8.53	2	0.27

TABLE 32

Total concentration and ratio of DNA:RNA:protein:carbohydrate:phospholipid for *P. halodurans* grown at 20 C and 200 rpm to the mid-logarithmic phase in modified 2216E medium with supplemental NaCl concentrations ranging from 0.0 to 3.45 M.

Supplemental NaCl, M	DNA µg/ml	DNA/DNA	RNA µg/ml	RNA/DNA	Protein µg/ml	Prot/DNA	Carbohydrate µg/ml	Carb/DNA	Phospholipid µg/ml	Phos/DNA
0.0	57	1.00	206	3.61	1004	17.61	478	8.39	21	0.37
0.0	60	1.00	213	3.55	1075	17.92	510	8.50	24	0.40
0.90	56	1.00	195	3.49	979	17.48	477	8.51	20	0.36
0.90	56	1.00	196	3.50	982	17.53	471	8.41	21	0.38
1.70	54	1.00	191	3.53	949	17.57	453	8.39	21	0.39
1.70	50	1.00	179	3.58	882	17.64	423	8.46	18	0.36
2.60	46	1.00	164	3.57	822	17.88	388	8.43	15	0.33
2.60	48	1.00	173	3.60	853	17.77	409	8.52	17	0.37
3.45	24	1.00	86	3.59	427	17.70	202	8.41	8	0.33
3.45	23	1.00	83	3.61	403	17.50	193	8.49	7	0.31

TABLE 33

Total concentration and ratio of DNA:RNA:protein:carbohydrate:phospholipid for P. halodurans grown at 20 C and 200 rpm to the late logarithmic phase in modified 2216E medium with supplemental NaCl concentrations ranging from 0.0 to 3.45 M.

Supplemental NaCl, M	DNA µg/ml	DNA/DNA	RNA µg/ml	RNA/DNA	Protein µg/ml	Prot/DNA	Carbohydrate µg/ml	Carb/DNA	Phospholipid µg/ml	Phos/DNA
0.0	125	1.00	501	4.01	2829	22.63	1506	12.05	70	0.56
0.0	118	1.00	476	4.03	2546	21.58	1429	12.11	71	0.60
0.90	111	1.00	452	4.07	2415	21.77	1330	11.98	59	0.53
0.90	112	1.00	450	4.02	2500	22.33	1307	11.67	62	0.55
1.70	107	1.00	427	3.99	2355	22.00	1260	11.78	60	0.56
1.70	110	1.00	444	4.04	2403	21.85	1308	11.89	64	0.58
2.60	95	1.00	386	4.06	2087	21.97	1128	11.87	56	0.59
2.60	89	1.00	360	4.05	1929	21.67	1060	11.91	46	0.52
3.45	43	1.00	172	4.00	925	21.58	513	11.93	25	0.57
3.45	46	1.00	186	4.05	1005	21.81	552	12.00	24	0.54

TABLE 34

Total concentration and ratio of DNA:RNA:protein:carbohydrate:phospholipid for P. halodurans grown at 20 C and 200 rpm to the stationary phase in modified 2216E medium with supplemental NaCl concentrations ranging from 0.0 to 3.45 M.

Supplemental	DNA		RNA		Protein		Carbohydrate		Phospholipid	
NaCl, M	µg/ml	DNA/DNA	µg/ml	RNA/DNA	µg/ml	Prot/DNA	µg/ml	Carb/DNA	µg/ml	Phos/DNA
0.0	167	1.00	579	3.47	4230	25.33	2390	14.31	109	0.65
0.0	175	1.00	614	3.56	4359	24.90	2473	14.13	116	0.66
0.90	158	1.00	529	3.35	3939	24.93	2244	14.20	92	0.58
0.90	161	1.00	549	3.41	4044	25.12	2300	14.29	97	0.60
1.70	146	1.00	508	3.48	3655	25.03	2044	14.00	80	0.55
1.70	143	1.00	495	3.46	3615	25.30	2026	14.17	82	0.57
2.60	128	1.00	435	3.38	3225	25.18	1792	14.01	81	0.63
2.60	131	1.00	457	3.46	3262	24.89	1860	14.18	86	0.66
3.45	63	1.00	219	3.45	1575	25.00	895	14.21	37	0.58
3.45	59	1.00	201	3.41	1492	25.28	837	14.19	35	0.61

TABLE 35

Percent chemical constituent of dry weight of cell envelopes extracted from P. halodurans grown at 20 C and 200 rpm to the early logarithmic (EL) and stationary phase (ST) in modified 2216E medium and modified 2216E medium supplemented with 1.70 and 3.45 M NaCl.

	Supplemental NaCl, M					
	0.0		1.70		3.45	
Chemical Constituent	EL	ST	EL	ST	EL	ST
DNA	ND	ND	ND	ND	ND	ND
RNA	ND	ND	ND	ND	ND	ND
Protein	73.6	74.5	74.5	74.9	73.3	73.9
Carbohydrate	20.7	21.1	20.0	20.2	20.5	20.5
Reducing Substance	1.7	1.8	1.5	1.6	1.7	1.7
Phospholipid	1.3	1.3	1.6	1.6	1.3	1.4
Total Lipid	5.8	5.3	6.0	5.9	5.8	5.7
Nitrogen	12.4	12.1	12.3	12.7	12.0	12.1
Phosphorous	1.0	0.9	1.1	0.9	1.0	1.1
Hexosamine	1.7	1.8	1.7	1.8	1.7	1.9

ND = Not Detected

TABLE 36

Amino acid composition (expressed as mM/mg cell envelope) of cell envelopes extracted from P. halodurans grown at 20 C and 200 rpm to the stationary phase in modified 2216E medium and modified 2216E medium supplemented with 1.70 and 3.45 M NaCl.

	Supplemental NaCl, M		
Amino Acid	0.0	1.70	3.45
Alanine	0.248	0.239	0.241
Arginine	0.101	0.103	0.108
Aspartic Acid	0.285	0.272	0.273
Cysteine	0.026	0.025	0.028
Glutamic Acid	0.046	0.048	0.045
Glycine	0.217	0.211	0.209
Histidine	0.032	0.030	0.029
Isoleucine	0.126	0.120	0.128
Leucine	0.205	0.208	0.202
Lysine	0.098	0.101	0.103
Methionine	0.027	0.026	0.029
Phenylalanine	0.127	0.129	0.122
Proline	0.090	0.088	0.085
Serine	0.177	0.171	0.169
Threonine	0.145	0.135	0.138
Tyrosine	0.089	0.082	0.090
Valine	0.126	0.121	0.122

Fig. 1. Seasonal variations of pH (⊡), salinity (△), and temperature (⊙) of water samples collected from the Great Bay estuarine complex on different sampling dates.

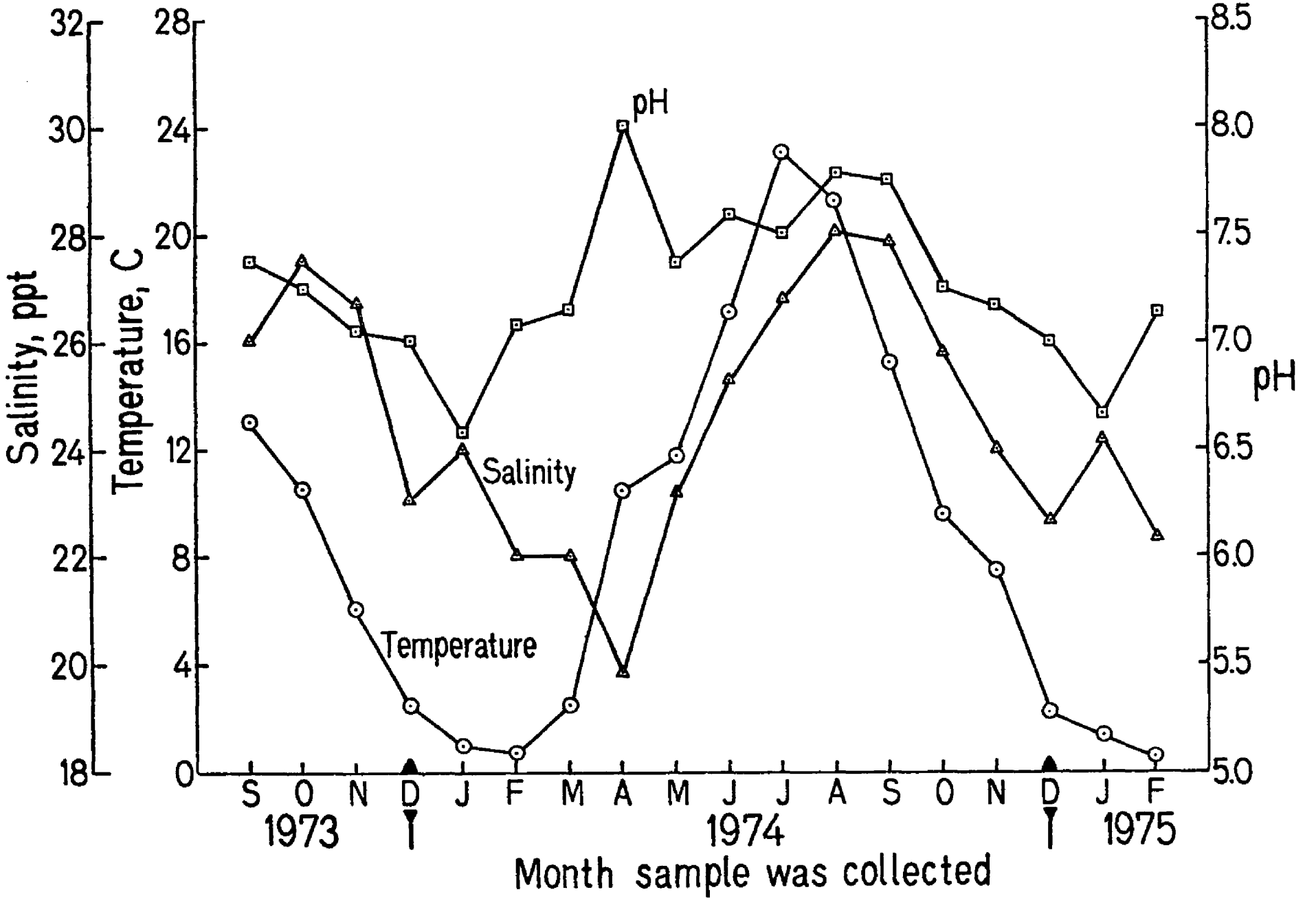

Salinity, ppt
Temperature, C
pH
32
30
28
26
24
22
20
18
28
24
20
16
12
8
4
0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
pH
Salinity
Temperature
S O N D J F M A M J J A S O N D J F
1973
1974
1975
Month sample was collected

Fig. 2. Colony forming units per ml (CFU/ml) of bacteria which developed from the Great Bay estuarine complex at 20 C after 14 days incubation on modified 2216E (Seven Seas) agar medium and the medium supplemented with NaCl concentrations ranging from 0.25 to 1.85 M on different sampling dates.

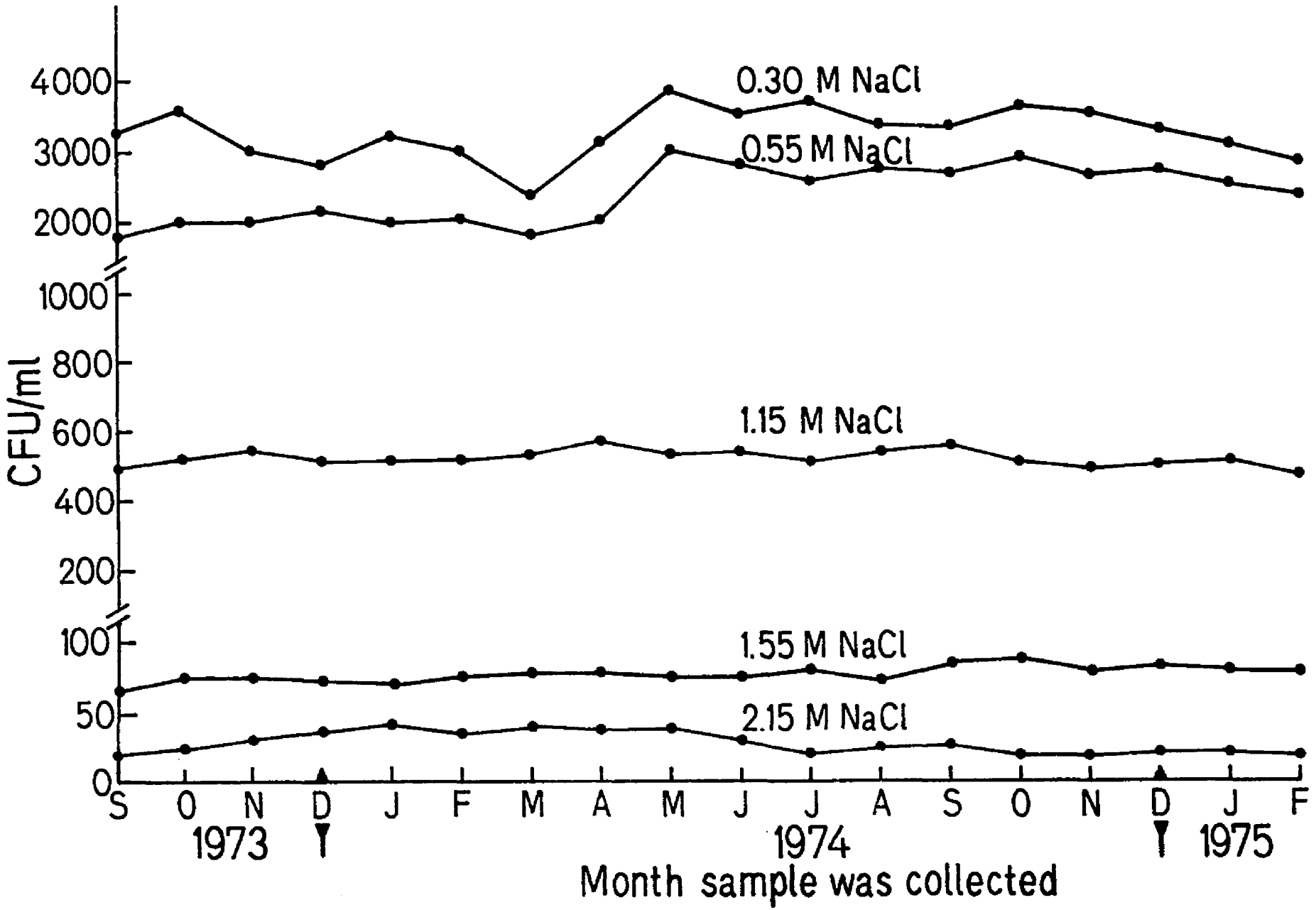

4000
3000
2000
1000
800
600
400
200
100
50
0
CFU/ml
0.30 M NaCl
0.55 M NaCl
1.15 M NaCl
1.55 M NaCl
2.15 M NaCl
S O N D J F M A M J J A S O N D J F
1973
1974
1975
Month sample was collected

Fig. 3. Colony forming units of bacteria which developed from the Great Bay estuarine complex at 20 C after 14 days incubation on modified 2216E (Seven Seas) agar medium and the medium supplemented with 1.00, 1.70, and 2.30 M NaCl on April 30, 1974.

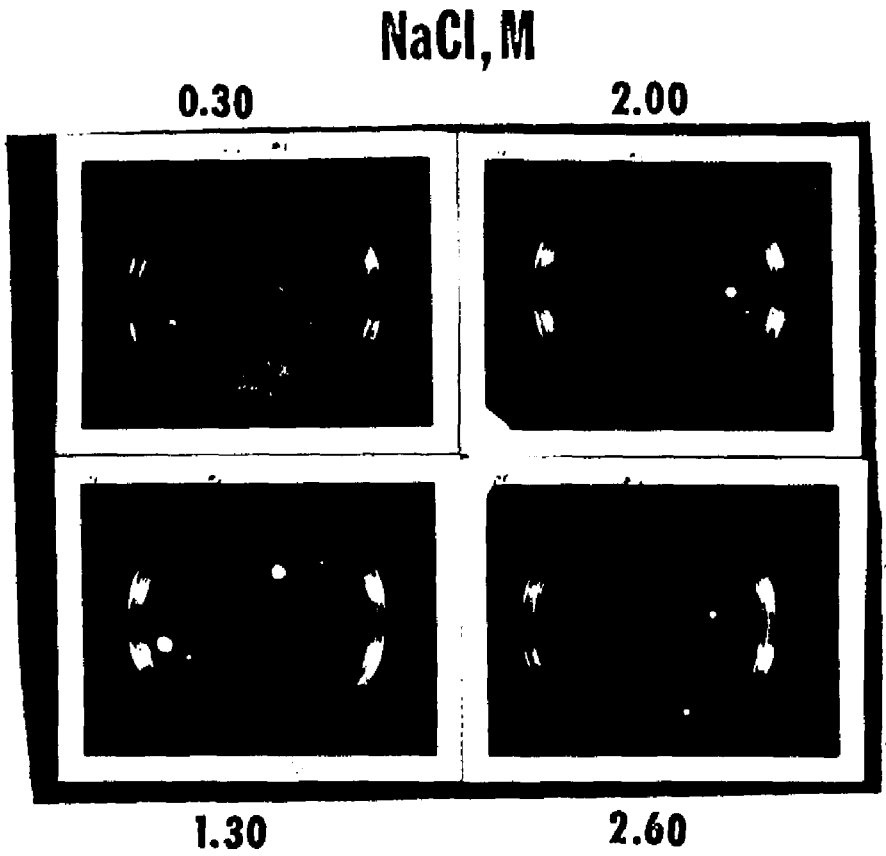

NaCl, M
0.30
2.00
1.30
2.60

Fig. 4. Phase contrast photomicrographs of halotolerant bacterial cells grown at 20 C and 200 rpm to the early logarithmic (3 h), mid-logarithmic (7 h), late logarithmic (12 h), and stationary (22 h) phase in modified 2216E medium.

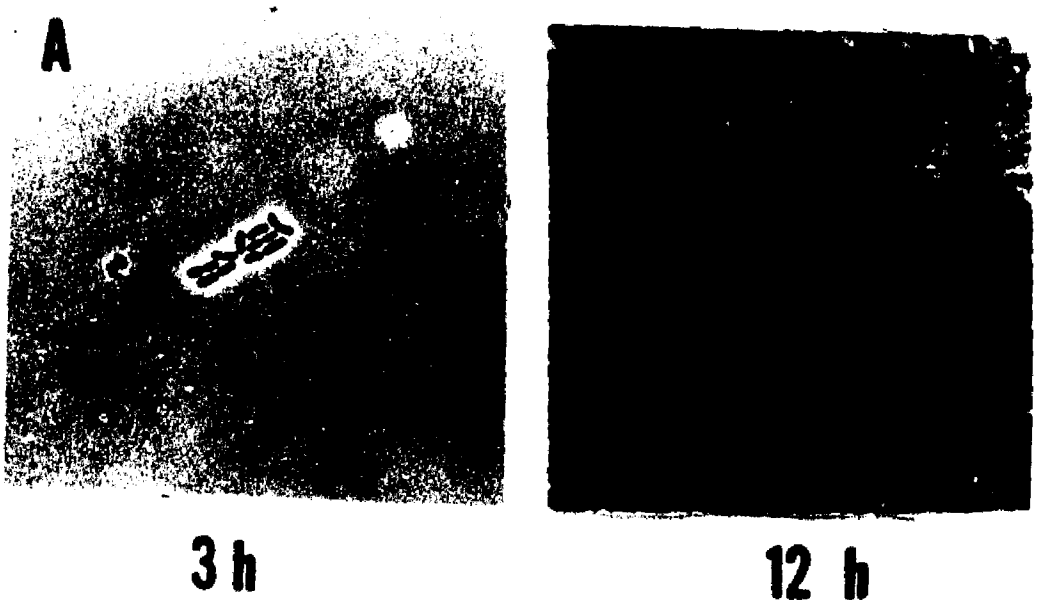
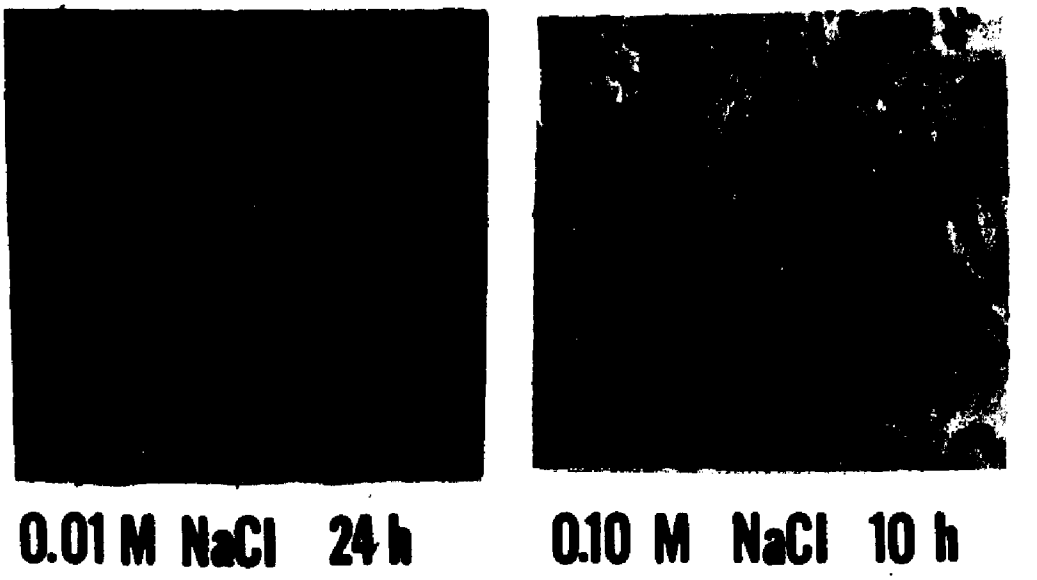
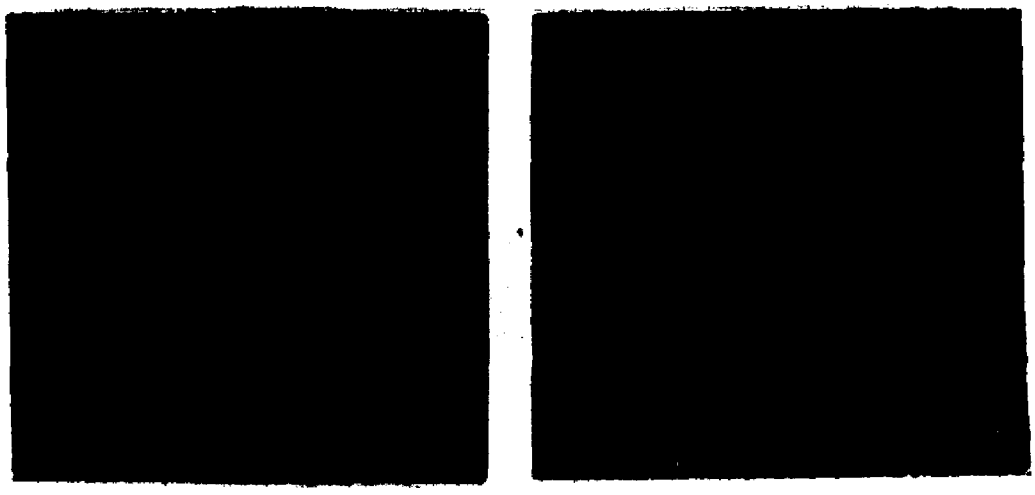

A
3 h
12 h
7 h
22 h
0.30 M NaCl
5.0 µm

Fig. 5. Phase contrast photomicrographs of halotolerant bacterial cells grown at 20 C and 200 rpm to the late logarithmic phase in modified 2216E medium with total NaCl concentrations of 0.01, 0.05, 0.10, and 0.30 M.



0.01 M NaCl 24 h

0.10 M NaCl 10 h



0.05 M NaCl 18 h

0.30 M NaCl 10 h

5.0 µm

Fig. 6. Phase contrast photomicrographs of bacterial cells grown at 20 C and 200 rpm to the late logarithmic phase in modified 2216E medium and the medium supplemented with NaCl concentrations ranging from 0.90 to 3.45 M NaCl.

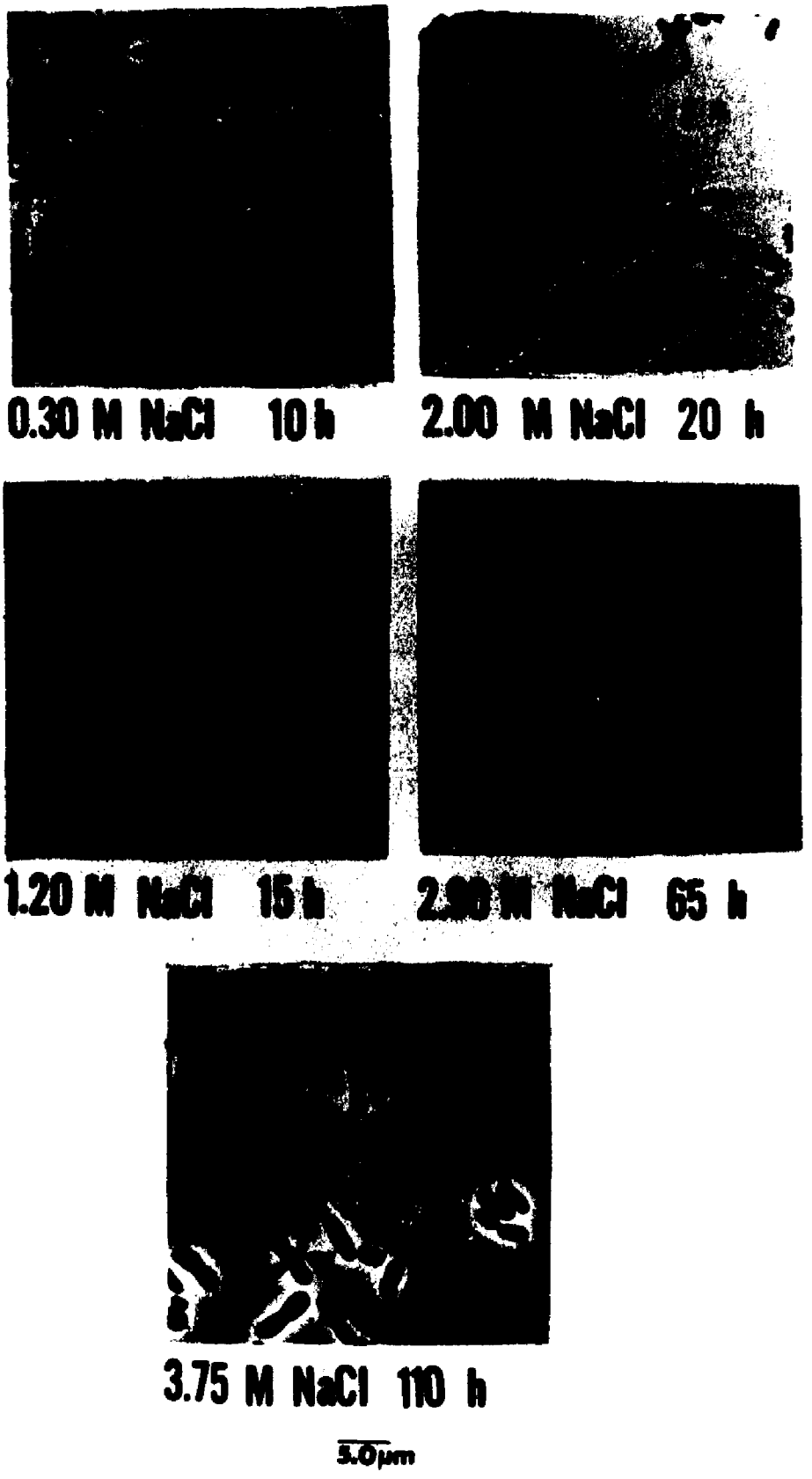
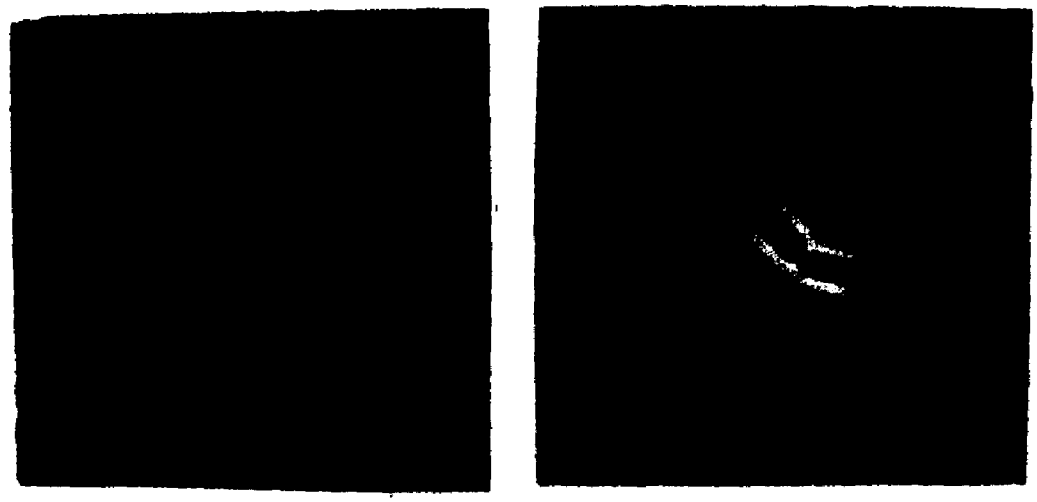

0.30 M NaCl 10 h
2.00 M NaCl 20 h
1.20 M NaCl 15 h
3.75 M NaCl 110 h
5.0μm

Fig. 7. Phase contrast photomicrographs of bacterial cells grown at 20 C and 200 rpm for 3 h in modified 2216E medium and stressed subsequently with 2.60 M NaCl for 12, 24, 48, and 72 h.

12 h

48 h



24 h

72 h

2.90 M NaCl

5.0 µm

Fig. 8. Transmission electron photomicrographs of bacterial cells grown at 20 C and 200 rpm to the late logarithmic phase in modified 2216E medium and the medium supplemented with NaCl concentrations ranging from 0.90 to 3.45 M. Cells were negatively stained with 0.5 % Na-phosphotungstate.

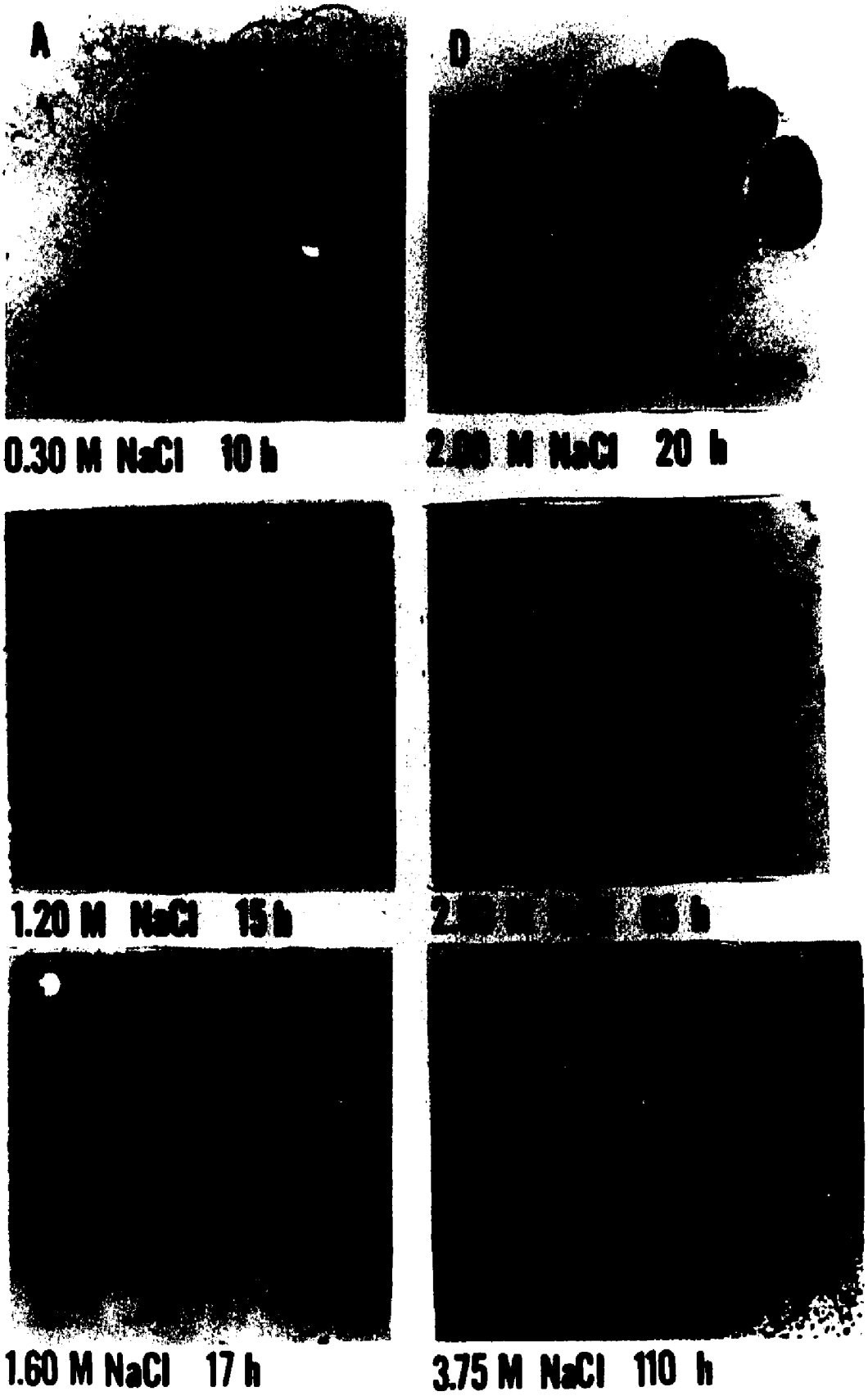
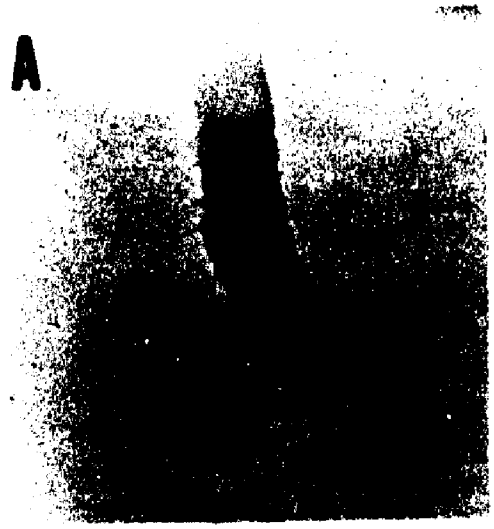
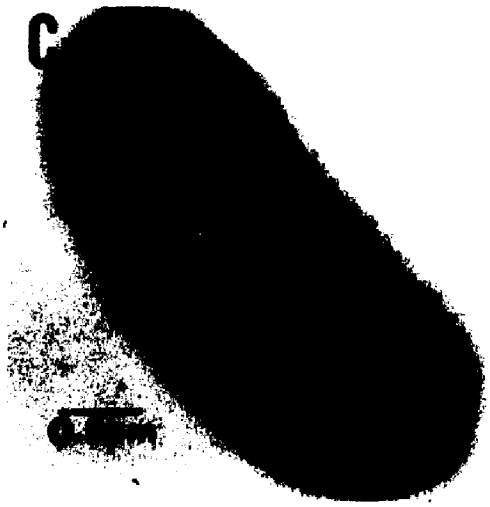

A
D
0.30 M NaCl 10 h
1.20 M NaCl 15 h
1.60 M NaCl 17 h
3.75 M NaCl 110 h

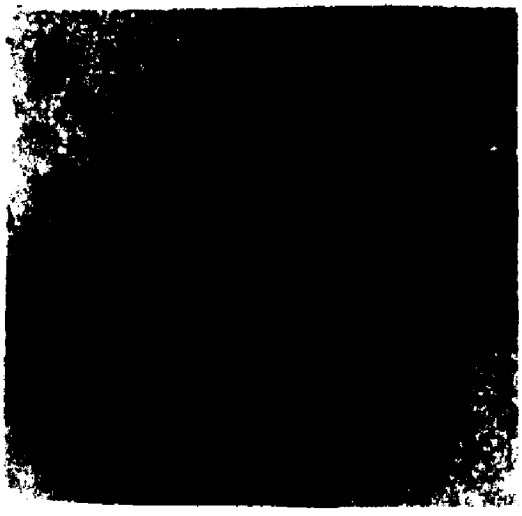
Fig. 9. Transmission electron photomicrographs of bacterial cells grown at 20 C and 200 rpm to the late logarithmic phase in modified 2216E medium and the medium supplemented with 1.70, 2.60, and 3.45 M NaCl. Cells were shadow cast with platinum metal.



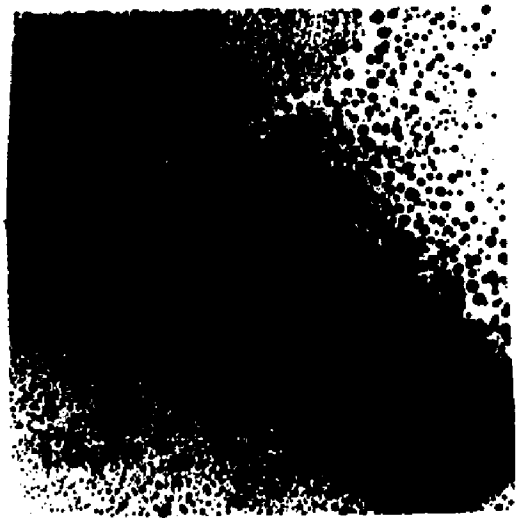
0.30 M NaCl 10 h



2.90 M NaCl 65 h



2.00 M NaCl 20 h



3.75 M NaCl 110 h

Fig. 10. Scanning electron photomicrographs of bacterial cells grown at 20 C and 200 rpm to the late logarithmic phase in modified 2216E medium and the medium supplemented with 2.60 M NaCl.

12,000X

25,000X

50,000X

0.30 M NaCl 10h

60,000X

2.90 M NaCl 65h

Fig. 11. Growth curve of P. halodurans grown at 20 C and 200 rpm in modified 2216E medium and the medium supplemented with NaCl concentrations ranging from 0.90 to 4.30 M.

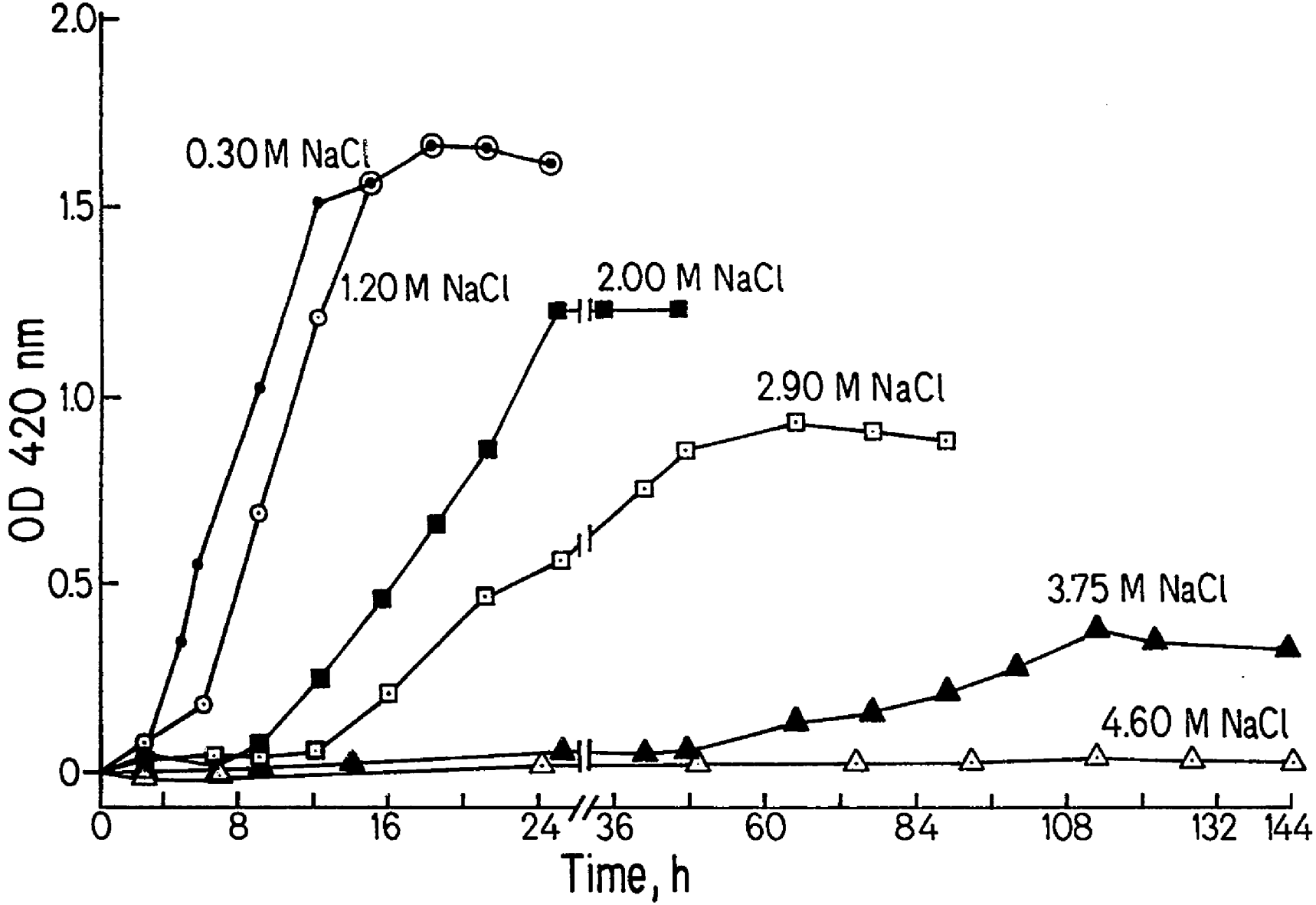

2.0
1.5
1.0
0.5
0
OD 420 nm
0.30 M NaCl
1.20 M NaCl
2.00 M NaCl
2.90 M NaCl
3.75 M NaCl
4.60 M NaCl
0
8
16
24
36
60
84
108
132
144
Time, h

Fig. 12. Cultural stages of growth of P. halodurans grown at 20 C and 200 rpm in modified 2216E medium (broken line). Aliquots (50 ml) were removed at times indicated and stressed with 2.60 M NaCl. Periodically, CFU/ml were enumerated at 20 C after 10 days incubation on modified 2216E agar medium.

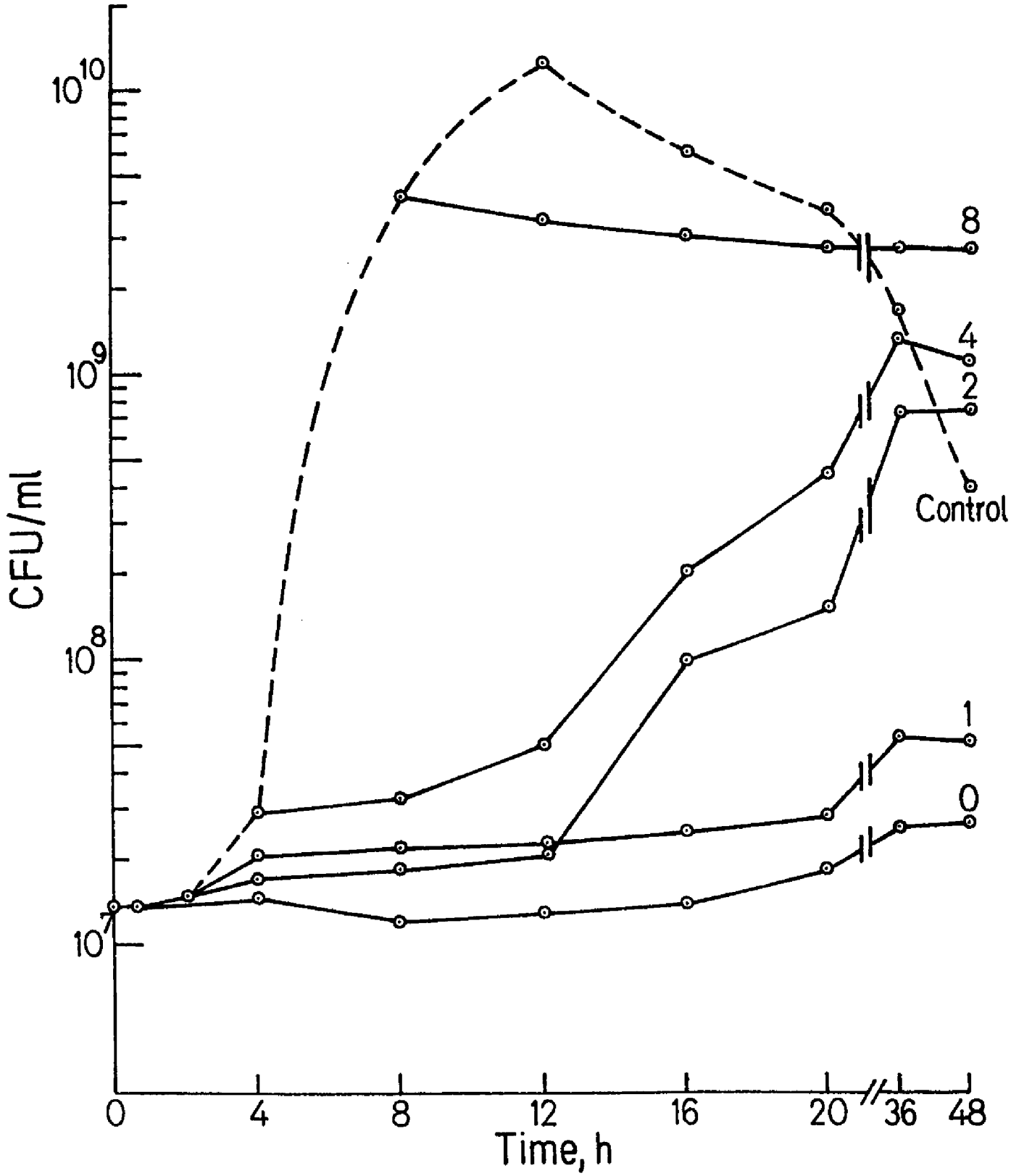

10^{10}
10^{9}
10^{8}
10^{7}
CFU/ml
8
4
2
Control
1
0
0
4
8
12
16
20
36
48
Time, h

Fig. 13. Specific growth rate of P. halodurans grown at temperatures ranging from 5 to 35 C and 200 rpm in modified 2216E medium containing 1.0 % nutrients with total NaCl concentrations ranging from 0.01 to 3.75 M.

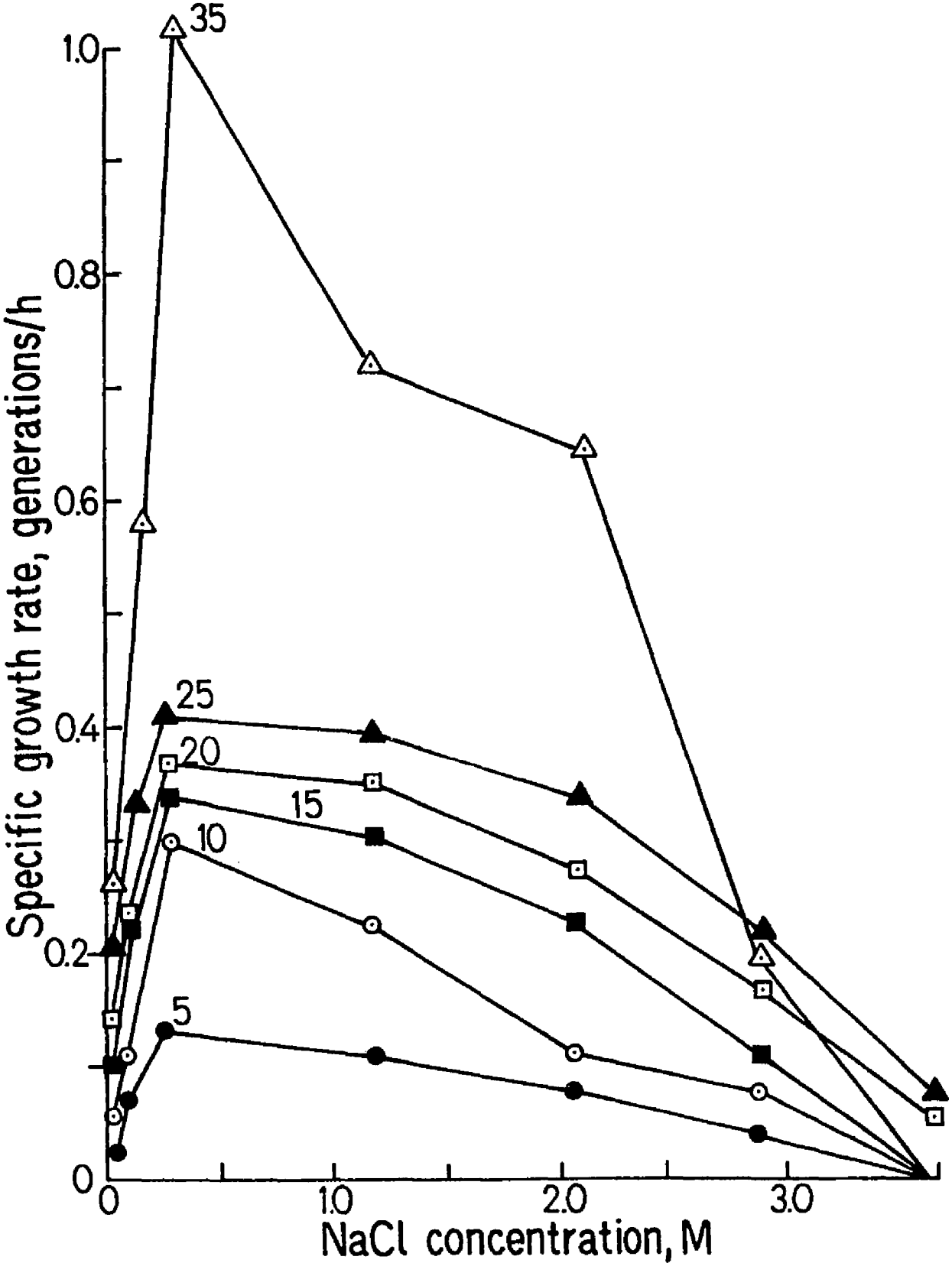

35
25
20
15
10
5
1.0
0.8
0.6
0.4
0.2
0
0
1.0
2.0
3.0
Specific growth rate, generations/h
NaCl concentration, M

Fig. 14. Specific growth rate of P. halodurans grown at temperatures ranging from 5 to 35 C and 200 rpm in modified 2216E medium containing 0.2 % nutrients with total NaCl concentrations ranging from 0.01 to 3.75 M.

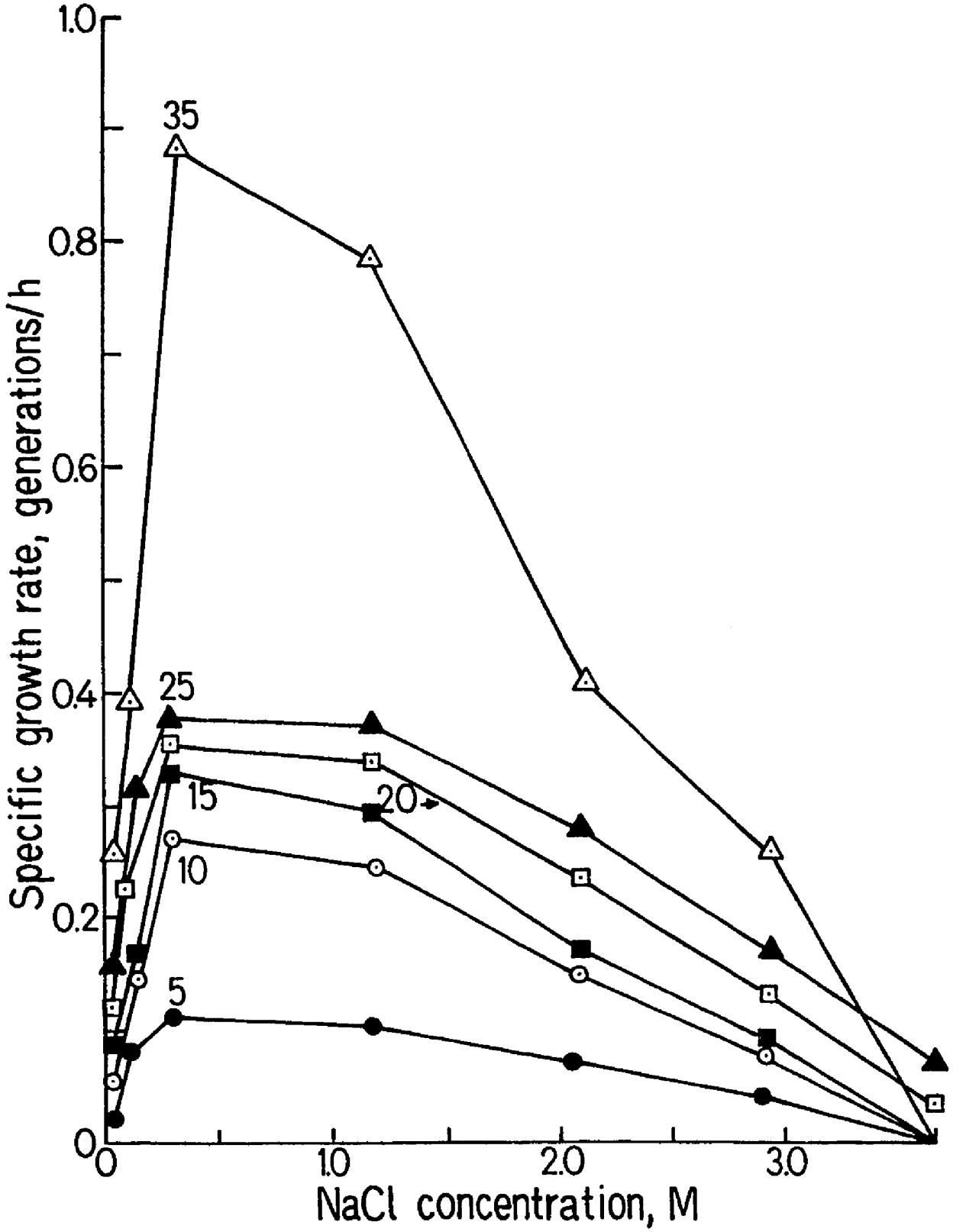

1.0
0.8
0.6
0.4
0.2
0
Specific growth rate, generations/h
35
25
15
20
10
5
0
1.0
2.0
3.0
NaCl concentration, M

Fig. 15. Specific growth rate of *P. halodurans* grown at temperatures ranging from 5 to 35 C and 200 rpm in modified 2216E medium containing 0.02 % nutrients with total NaCl concentrations ranging from 0.01 to 3.75 M.

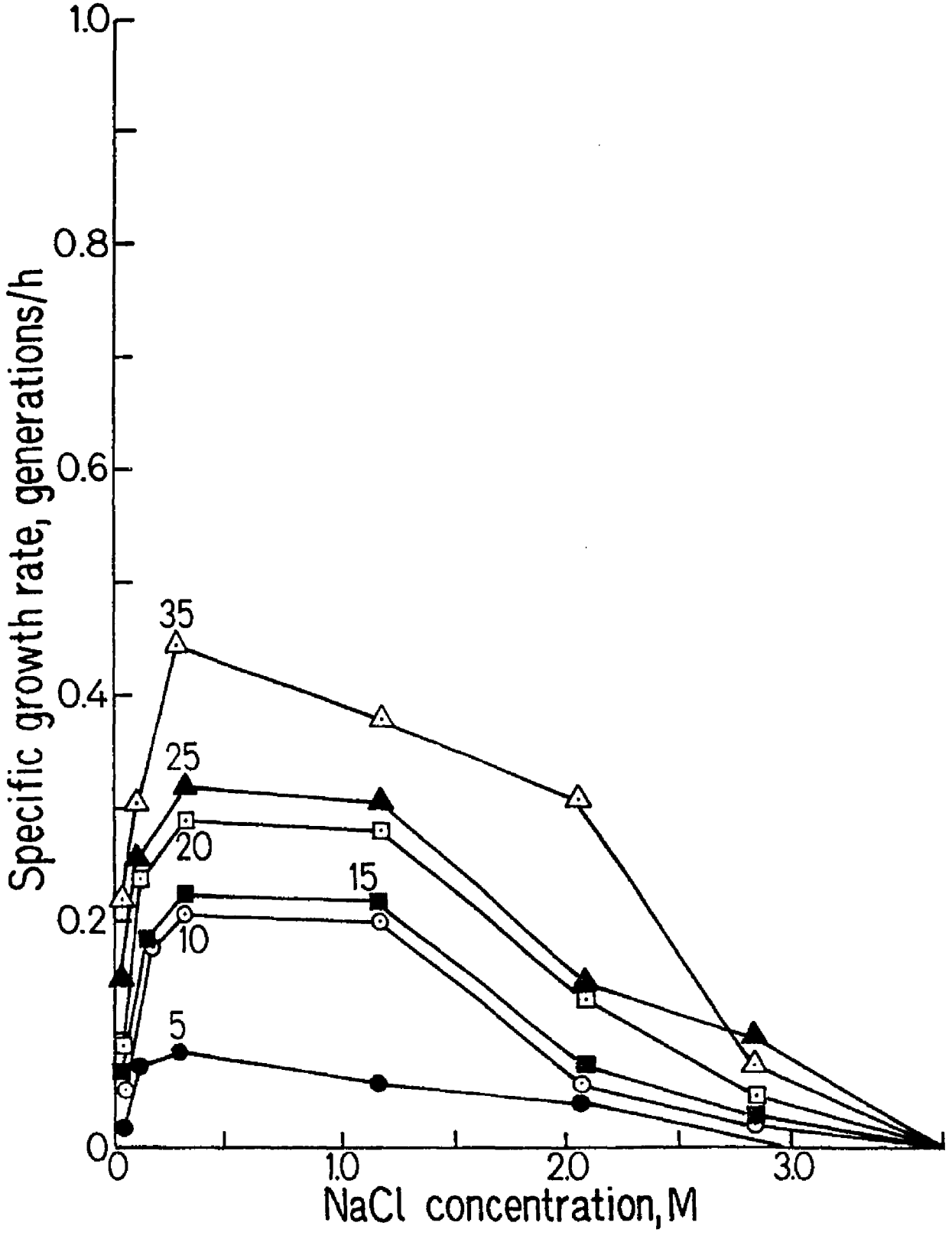

1.0
0.8
0.6
0.4
0.2
0
Specific growth rate, generations/h
35
25
20
15
10
5
0
1.0
2.0
3.0
NaCl concentration, M

Fig. 16. Maximum optical density attained by P. halodurans grown at 20 C and 200 rpm in modified 2216E medium with total NaCl concentrations ranging from 0.00 to 4.10 M.

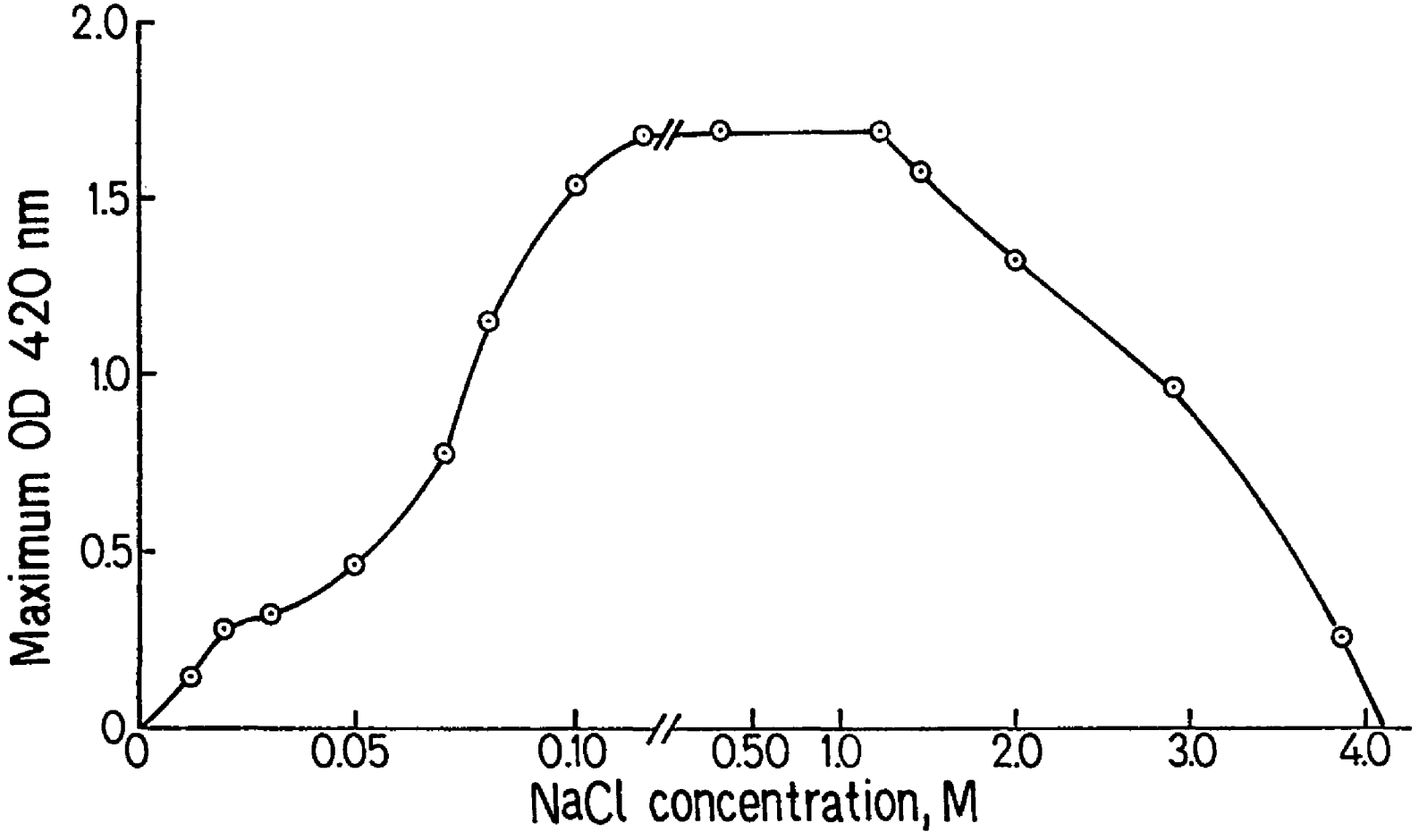

Maximum OD 420 nm
2.0
1.5
1.0
0.5
0
0
0.05
0.10
0.50
1.0
2.0
3.0
4.0
NaCl concentration, M

Fig. 17. Oxygen utilization by P. halodurans at 20 C in modified 2216E medium with total NaCl concentrations ranging from 0.01 to 5.45 M.

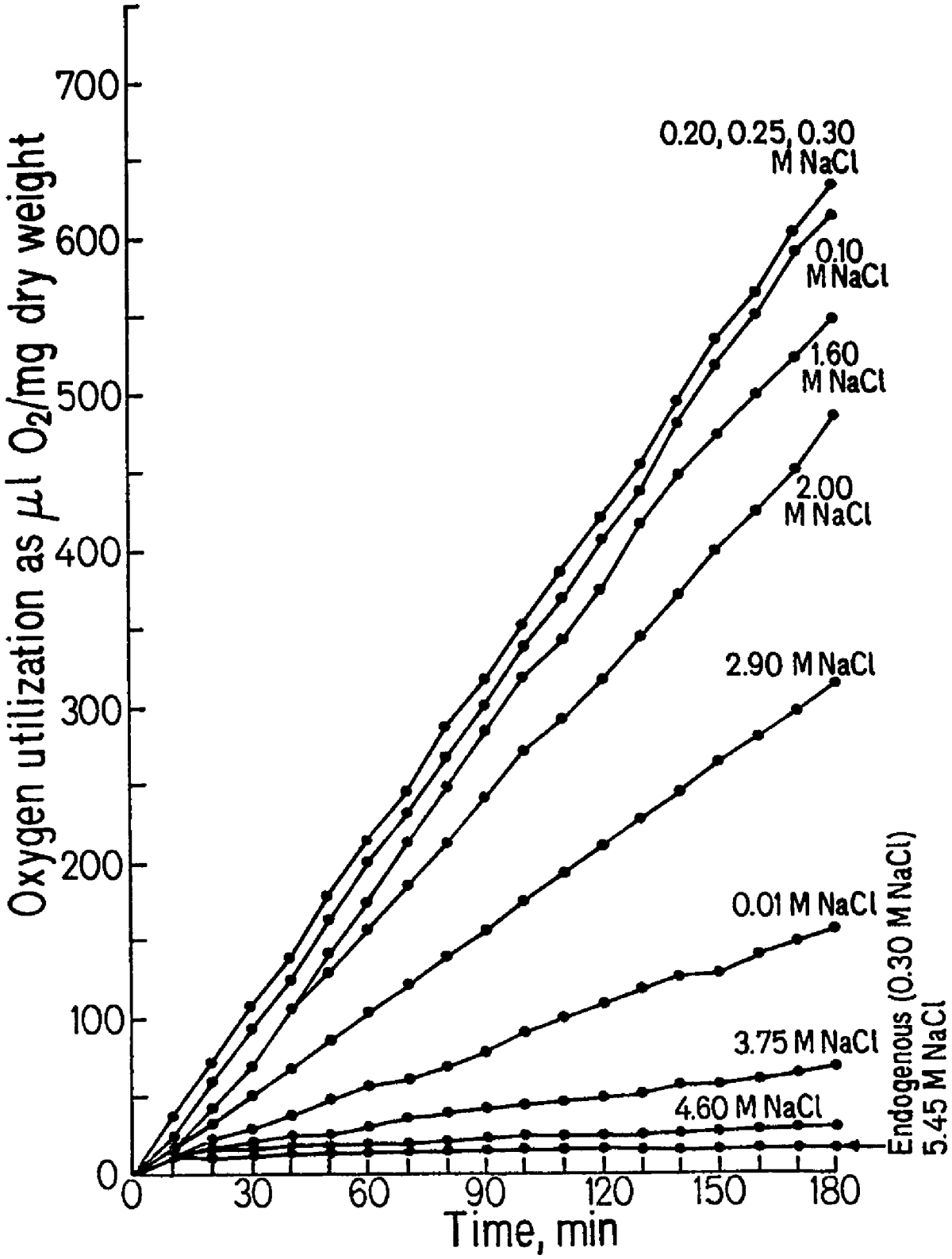

Oxygen utilization as μl O2/mg dry weight
700
600
500
400
300
200
100
0
0
30
60
90
120
150
180
Time, min
0.20, 0.25, 0.30 M NaCl
0.10 M NaCl
1.60 M NaCl
2.00 M NaCl
2.90 M NaCl
0.01 M NaCl
3.75 M NaCl
4.60 M NaCl
Endogenous (0.30 M NaCl)
5.45 M NaCl

Fig. 18. Oxygen utilization by P. halodurans grown initially at 20 C and 200 rpm to the late logarithmic phase in modified 2216E medium supplemented with 1.70 M NaCl and studied subsequently at 20 C in modified 2216E medium with total NaCl concentrations ranging from 0.20 to 4.60 M.

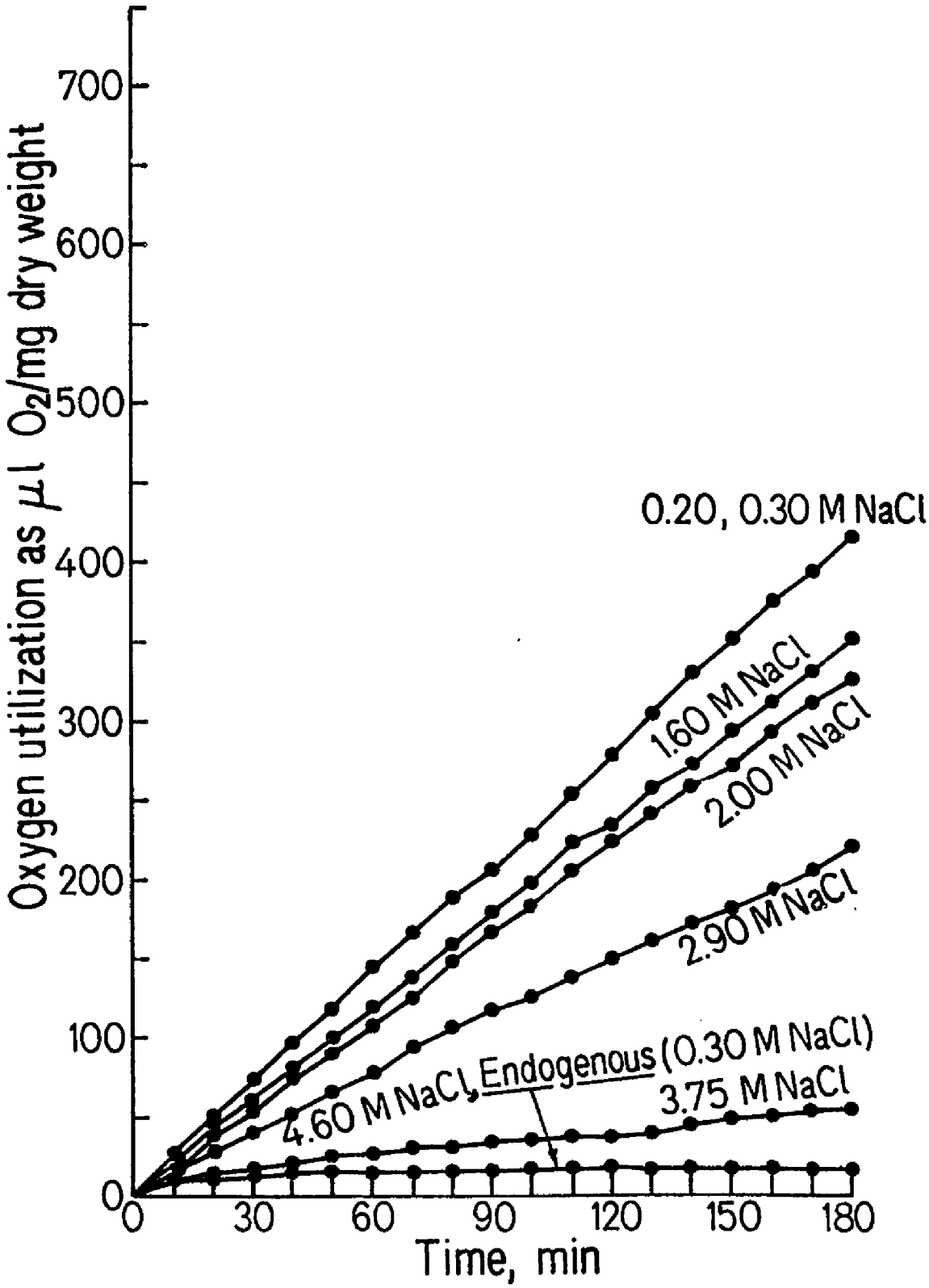

Oxygen utilization as μl O2/mg dry weight
700
600
500
400
300
200
100
0
0
30
60
90
120
150
180
Time, min
0.20, 0.30 M NaCl
1.60 M NaCl
2.00 M NaCl
2.90 M NaCl
4.60 M NaCl, Endogenous (0.30 M NaCl)
3.75 M NaCl

Fig. 19. Oxygen utilization by P. halodurans grown initially at 20 C and 200 rpm to the late logarithmic phase in modified 2216E medium supplemented with 2.60 M NaCl and studied subsequently at 20 C in modified 2216E medium with total NaCl concentration ranging from 0.20 to 4.60 M.

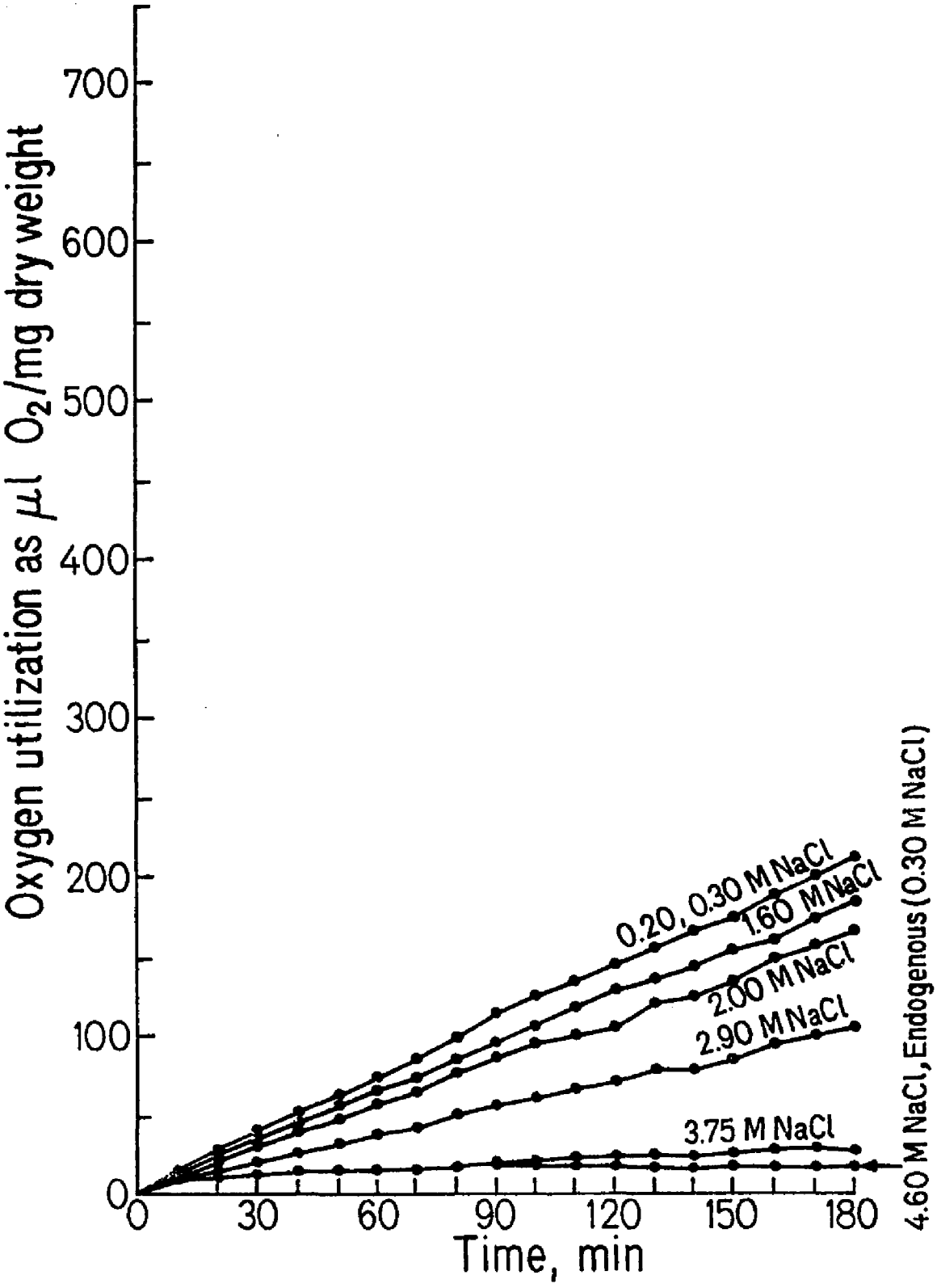

Oxygen utilization as μl O2/mg dry weight
700
600
500
400
300
200
100
0
0
30
60
90
120
150
180
Time, min
0.20, 0.30 M NaCl
1.60 M NaCl
2.00 M NaCl
2.90 M NaCl
3.75 M NaCl
4.60 M NaCl, Endogenous (0.30 M NaCl)

Fig. 20. Reduction of triphenyltetrazolium chloride (TTC) by P. halodurans incubated at 20 C in modified 2216E medium with total NaCl concentrations ranging from 0.01 to 3.75 M NaCl.

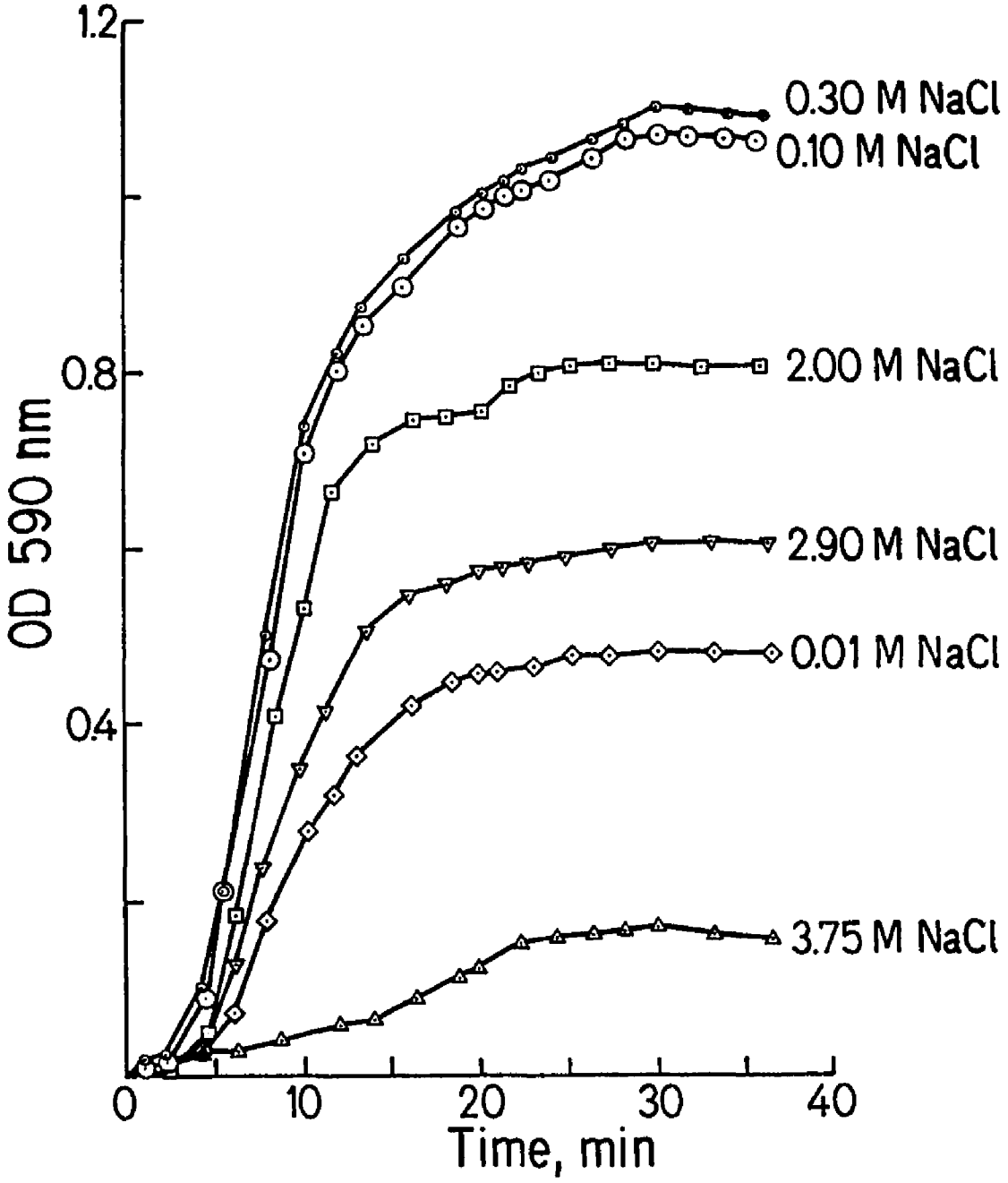

1.2
0.8
0.4
OD 590 nm
0.30 M NaCl
0.10 M NaCl
2.00 M NaCl
2.90 M NaCl
0.01 M NaCl
3.75 M NaCl
0
10
20
30
40
Time, min

Fig. 21. Reduction of dichloroindophenol (DCIP) by P. halodurans incubated at 20 C in modified 2216E medium with total NaCl concentrations ranging from 0.01 to 3.75 M NaCl.

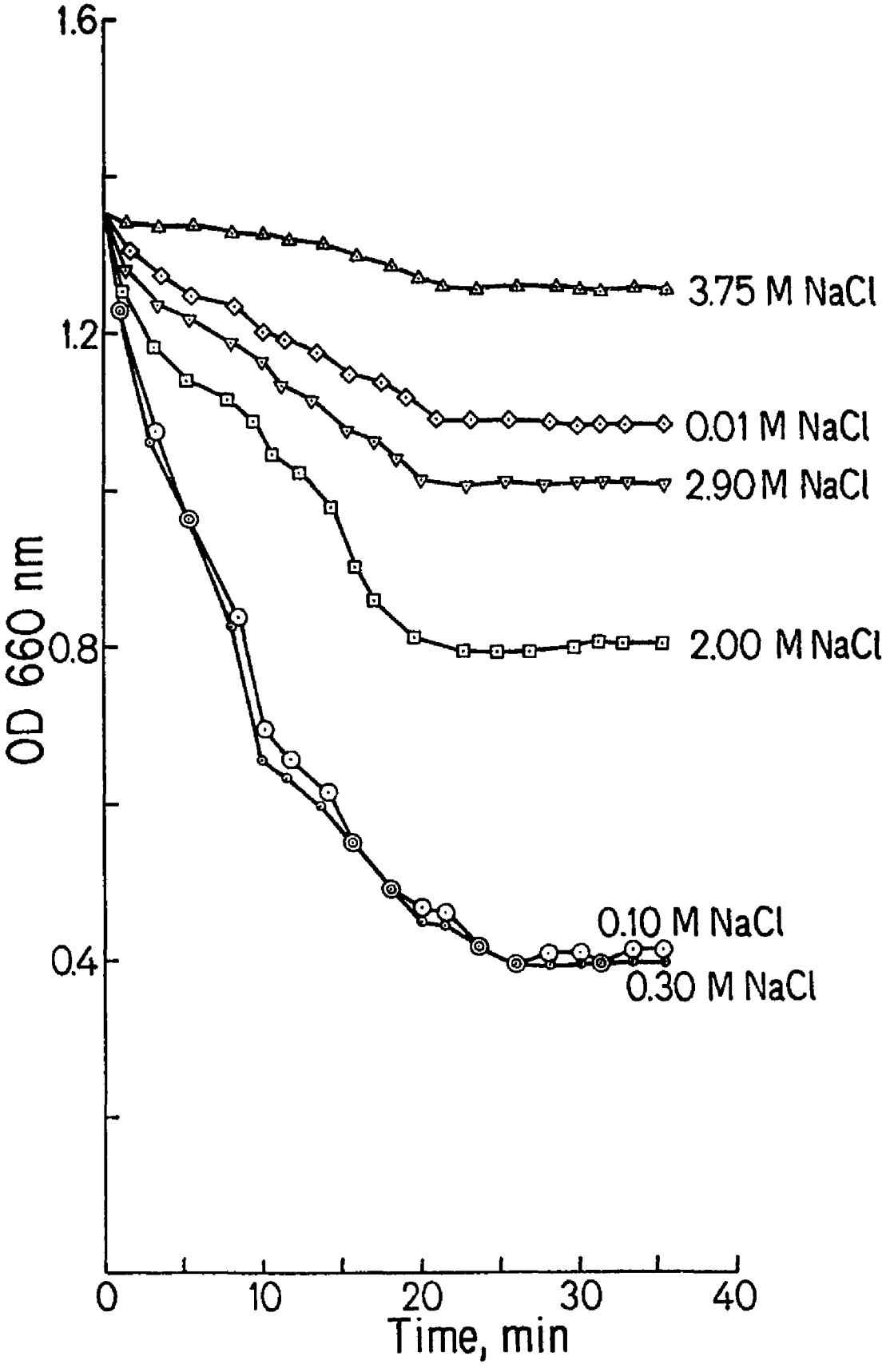

1.6
1.2
0.8
0.4
OD 660 nm
0
10
20
30
40
Time, min
3.75 M NaCl
0.01 M NaCl
2.90 M NaCl
2.00 M NaCl
0.10 M NaCl
0.30 M NaCl

Fig. 22. Reduction of methylene blue (MB) by P. halodurans incubated at 20 C in modified 2216E medium with total NaCl concentrations ranging from 0.01 to 3.75 M NaCl.

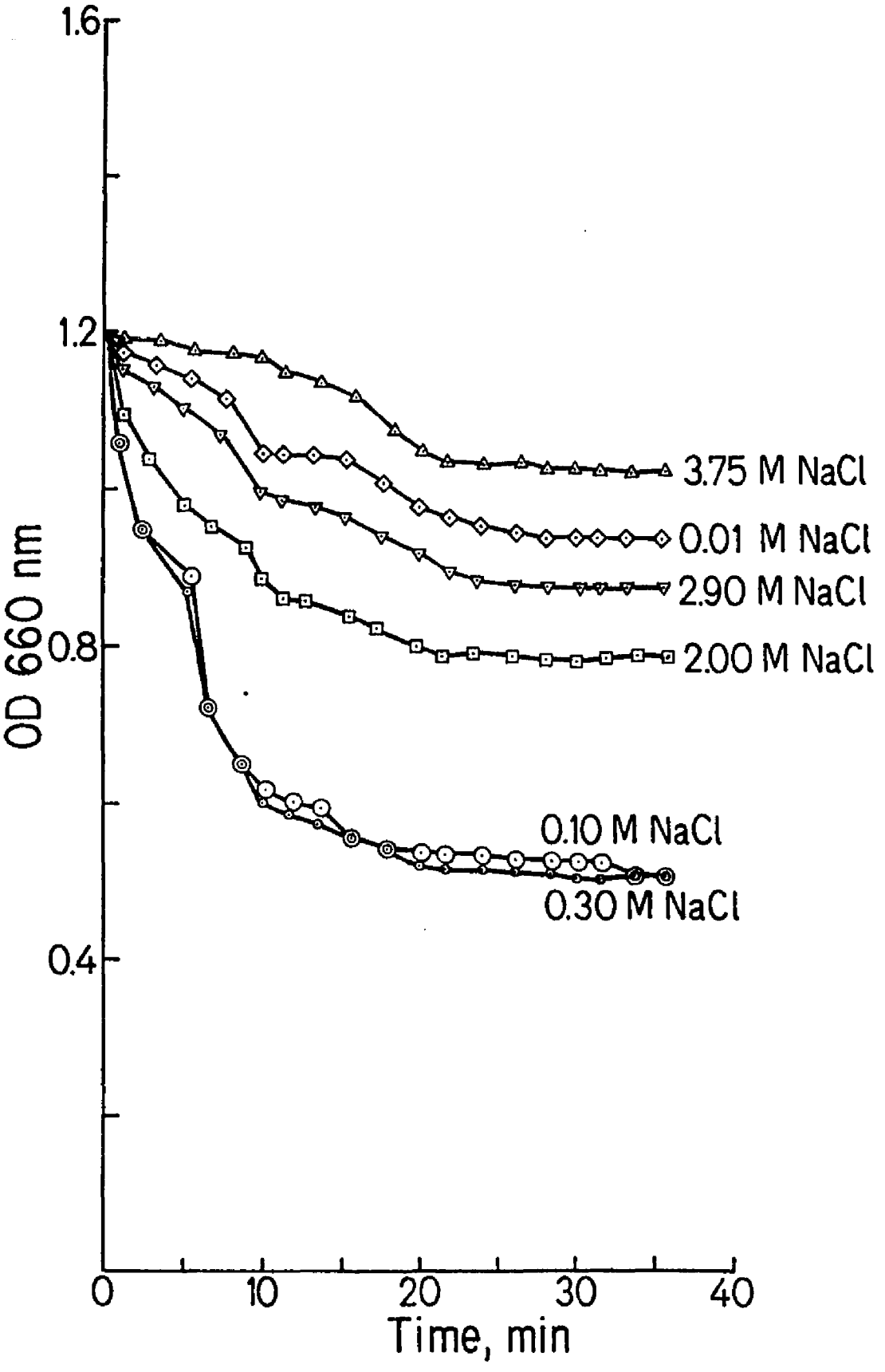

1.6
1.2
0.8
0.4
OD 660 nm
0
10
20
30
40
Time, min
3.75 M NaCl
0.01 M NaCl
2.90 M NaCl
2.00 M NaCl
0.10 M NaCl
0.30 M NaCl

Fig. 23. Absorption spectrum of the reduced cytochrome from whole cell extracts of P. halodurans grown at 20 C and 200 rpm to the late logarithmic phase in modified 2216E medium (A,B,C) and medium supplemented with 2.60 M NaCl (A',B',C').

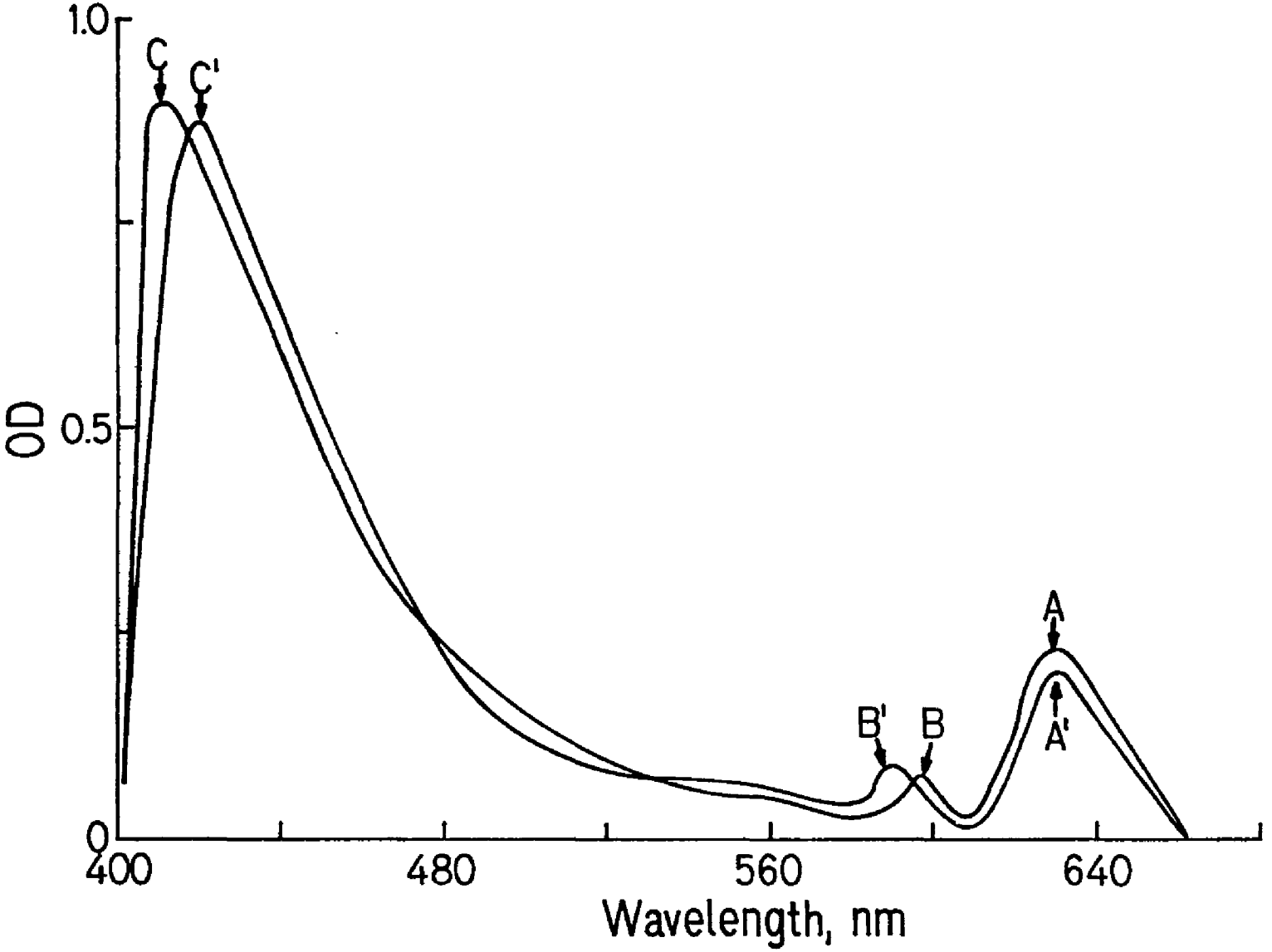

1.0
0.5
0
OD
C
C'
B'
B
A
A'
400
480
560
640
Wavelength, nm

Fig. 24. Difference spectrum of the oxidized and then reduced cytochromes from whole cell extracts of P. halodurans grown at 20 C and 200 rpm to the late logarithmic phase in modified 2216E medium (A,B,C,D) and medium supplemented with 2.60 M NaCl (A',B', C',D').

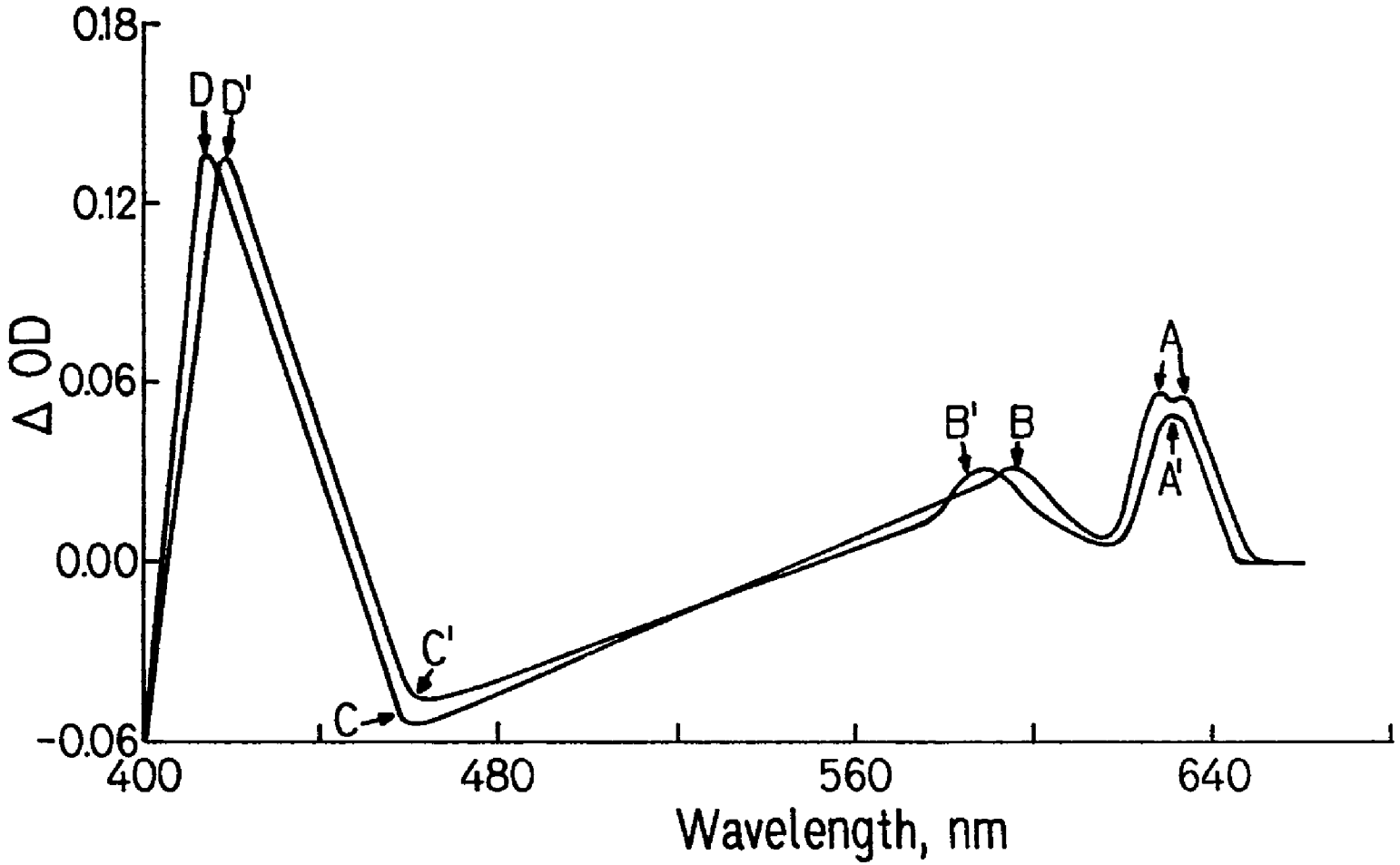

0.18
0.12
0.06
0.00
-0.06
Δ OD
D
D'
C'
C
B'
B
A
A'
400
480
560
640
Wavelength, nm

Fig. 25. ^{22}Na associated with the cell pellets (•) and growth (⊙) of P. halodurans at 20 C and 200 rpm in: (A) modified 2216E medium and the medium stressed with 2.60 M supplemental NaCl and 0.39 μCi/ml $^{22}NaCl$ after attaining an optical density of 0.25 (arrows), (B) modified 2216E medium supplemented with 1.30 M NaCl, and (C) modified 2216E medium supplemented with 2.60 M NaCl.

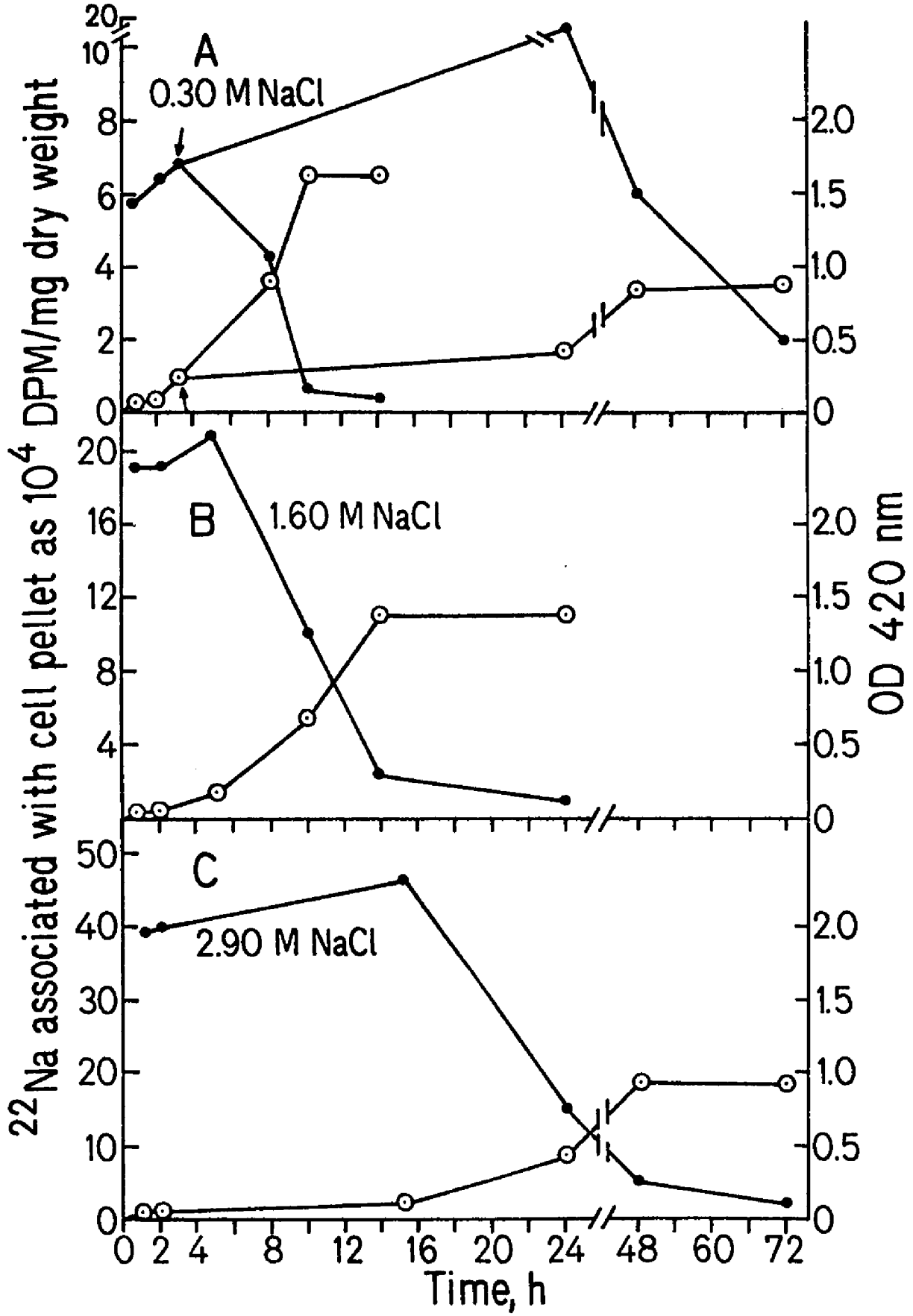

A
0.30 M NaCl
B
1.60 M NaCl
C
2.90 M NaCl
^{22}Na associated with cell pellet as 10^4 DPM/mg dry weight
OD 420 nm
Time, h
0 2 4 8 12 16 20 24 48 60 72

Fig. 26. C:N:P ratios of P. halodurans grown at 20 C and 200 rpm for 3 h in modified 2216E medium with aliquots removed and stressed with 2.60 and 4.30 M NaCl. (A) CFU/ml enumerated at 20 C after 10 days incubation on modified 2216E agar medium and (B) Relative C:P ratios (upper set of curves) and N:P ratios (lower set of curves).

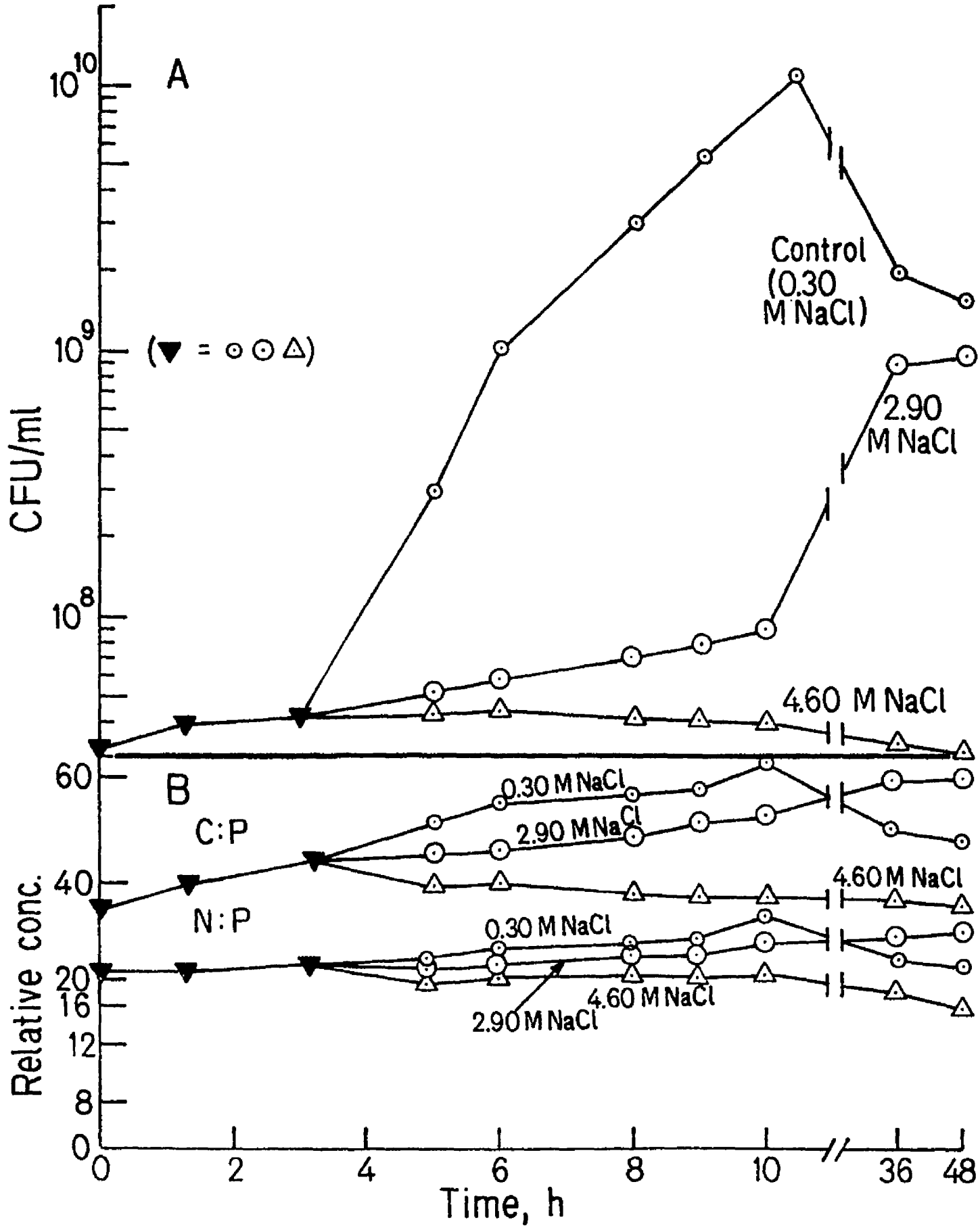

A
10^{10}
10^{9}
10^{8}
(▼ = ⊙ ⊙ △)
CFU/ml
Control (0.30 M NaCl)
2.90 M NaCl
4.60 M NaCl
B
C:P
N:P
0.30 M NaCl
2.90 M NaCl
4.60 M NaCl
0.30 M NaCl
2.90 M NaCl
4.60 M NaCl
Relative conc.
60
40
20
16
12
8
0
0
2
4
6
8
10
36
48
Time, h

Fig. 27. Ultraviolet spectrum of the cultural supernates from P. halodurans cells grown at 20 C and 200 rpm to the early logarithmic (EL) and stationary (ST) phase in modified 2216E medium and the medium supplemented with 1.70 and 3.45 M NaCl.

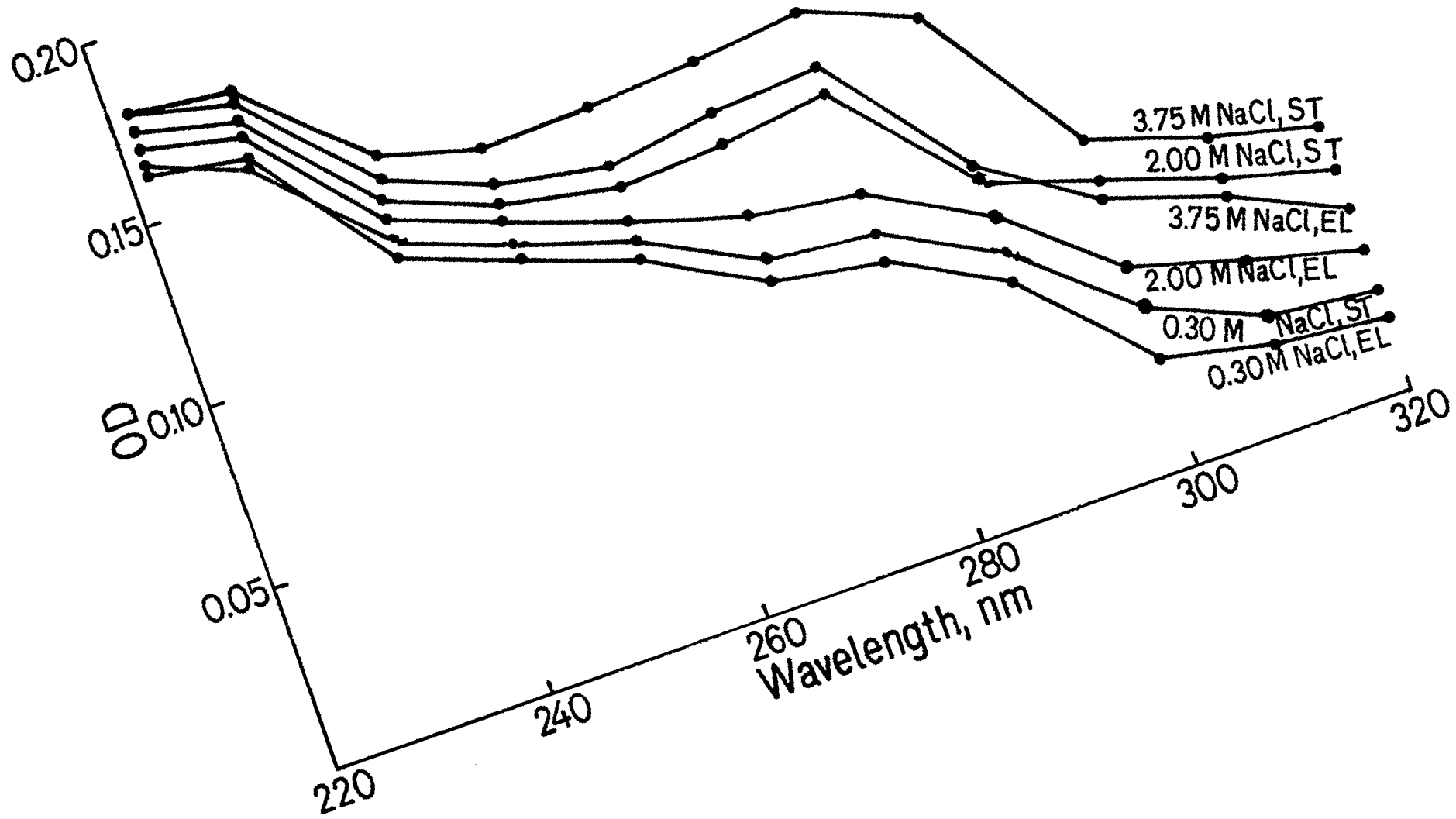
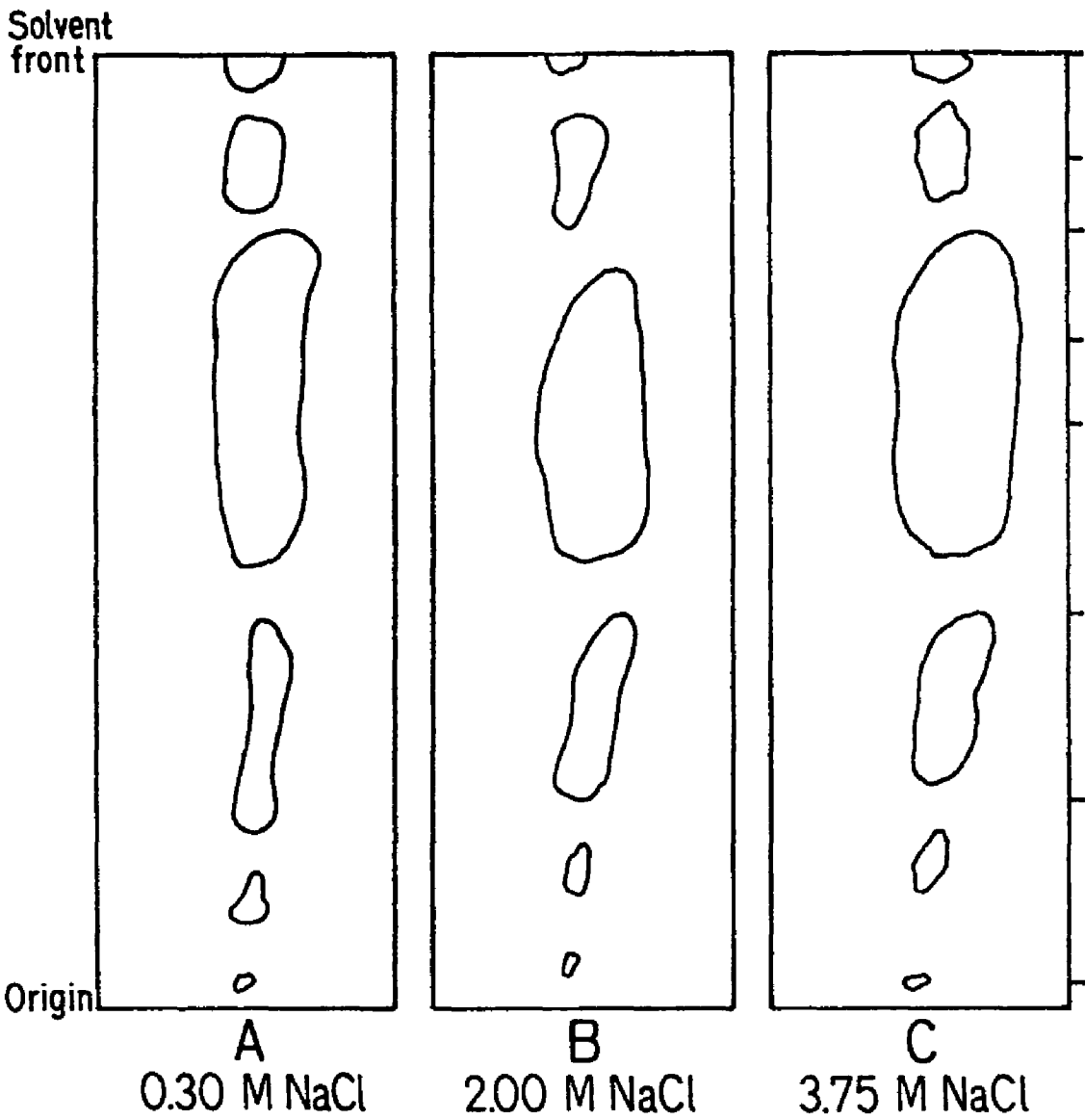

0.20
0.15
0.10
0.05
OD
220
240
260
280
300
320
Wavelength, nm
3.75 M NaCl, ST
2.00 M NaCl, ST
3.75 M NaCl, EL
2.00 M NaCl, EL
0.30 M NaCl, ST
0.30 M NaCl, EL

Fig. 28. Tracings of thin-layer chromatograms by thin-layer chromatography of cell envelope phospholipids of P. halodurans cells grown at 20 C and 200 rpm to the stationary phase in modified 2216E medium and the medium supplemented with 1.70 and 3.45 M NaCl. The Rf values of the standards were: phosphatidic acid (PA), 0.94; disphosphatidylglycerol (DPG), 0.84; phosphatidylethanolamine (PE), 0.65; phosphatidylserine (PS), 0.34; and phosphatidylcholine (PC), 0.18.



Rf	Visualization ninhydrin			I_2			Molybdate blue			Possible identity		
	A	B	C	A	B	C	A	B	C	A	B	C
1.0	-	-	-	-	-	-	+	+	+	PA	PA	PA
	-	-	-	+	+	+	+	+	+	DPG	DPG	DPG
0.8												
	+	+	+	+	+	+	+	+	+	PE	PE	PE
0.6												
0.4												
	+	+	+	+	+	+	+	+	+	PS	PS	PS
0.2												
	+	+	+	+	+	+	+	+	+	PC	PC	PC
0.0	+	+	+	+	+	+	+	+	+	?	?	?

Fig. 29. Transmission electron photomicrographs of thin sections of cell envelopes of P. halodurans grown at 20 C and 200 rpm to the stationary phase in modified 2216E medium and the medium supplemented with 1.70 and 3.45 M NaCl. Cell envelopes were fixed with 3 % glutaraldehyde.

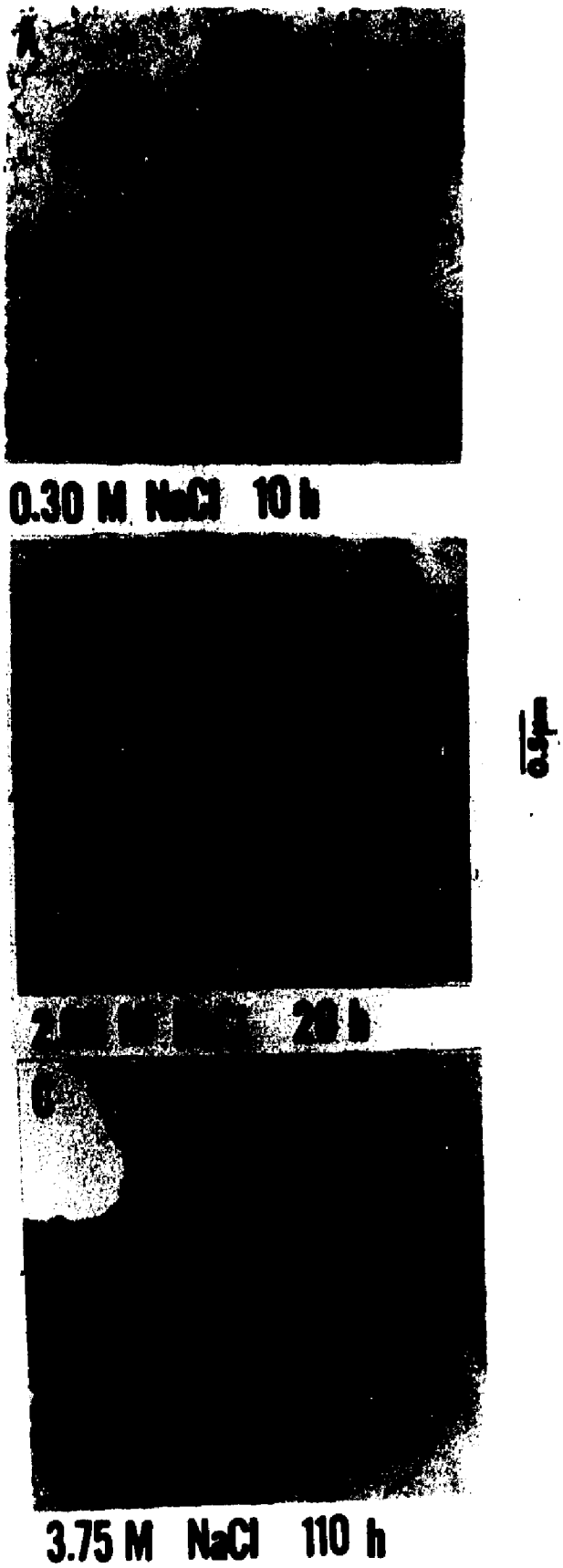
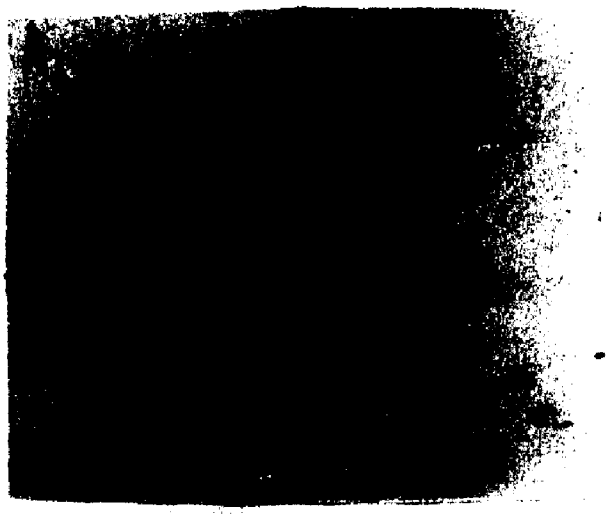
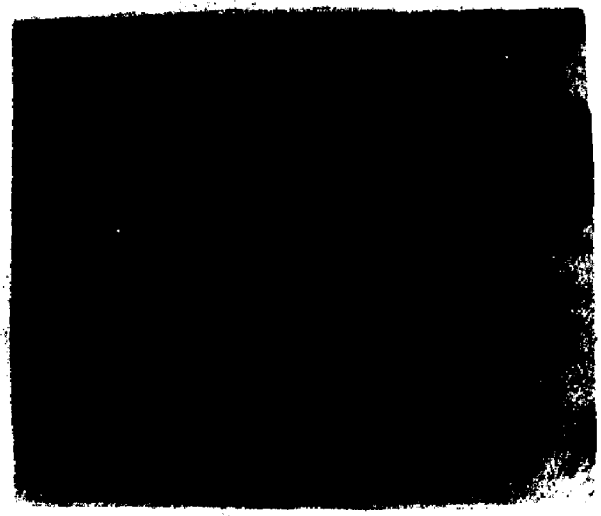

0.30 M NaCl 10 h
0.5 μm
3.75 M NaCl 110 h

Fig. 30. Transmission electron photomicrographs of thin sections of P. halodurans cells grown at 20 C and 200 rpm to the stationary phase in modified 2216E medium and the medium supplemented with 1.70 and 3.45 M NaCl. Cells were fixed with 3 % glutaraldehyde.



0.30 M NaCl 10 h

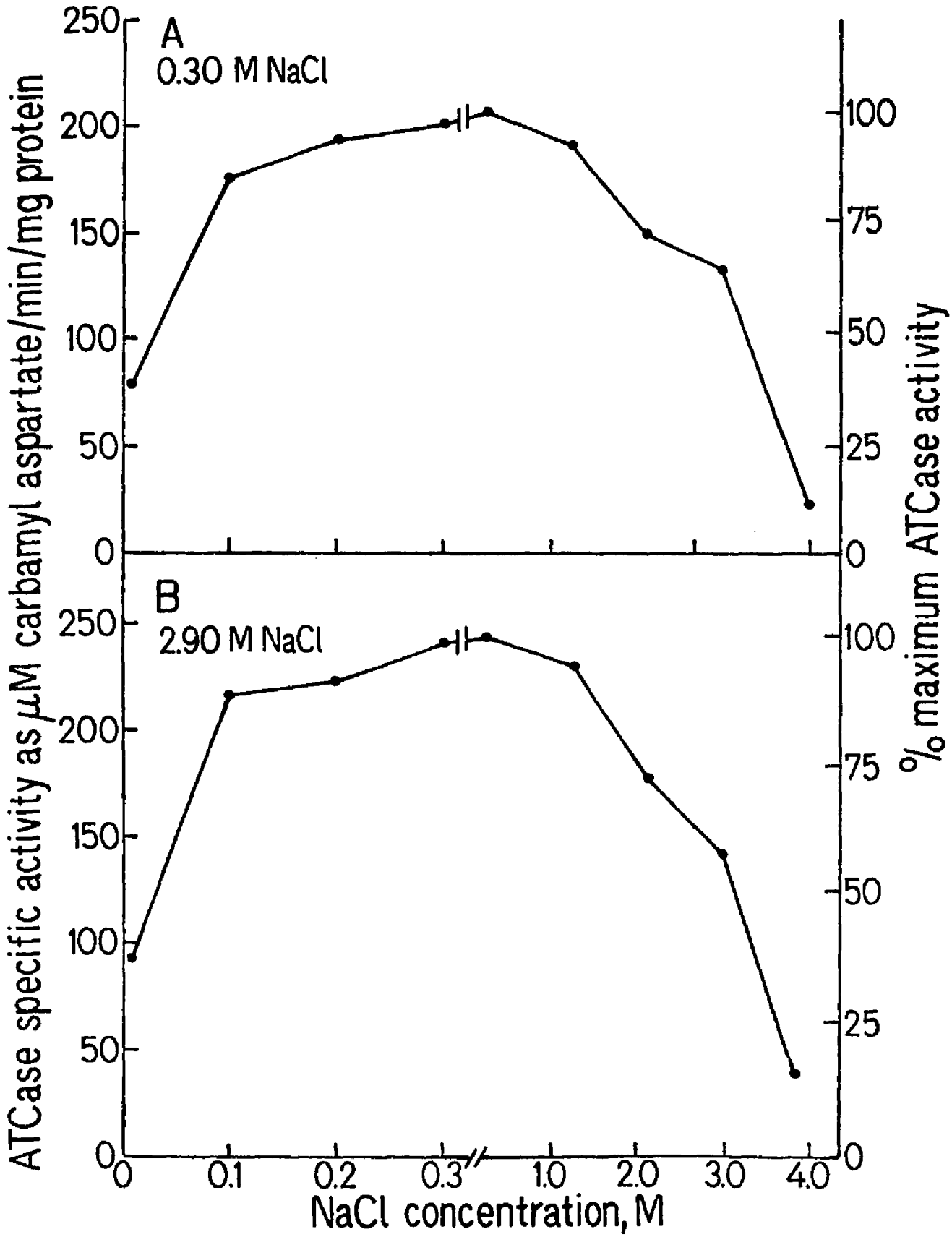


0.5 μm

2.00 M NaCl 20 h

3.75 M NaCl 110 h

Fig. 31. Specific activity of aspartate transcarbamylase (ATCase) isolated as a crude extract from P. halodurans cells grown at 20 C and 200 rpm to the stationary phase in: (A) modified 2216E medium and (B) modified 2216E medium containing 2.90 M NaCl and measured at 20 C in modified 2216E medium with total NaCl concentrations ranging from 0.01 to 3.75 M.


A
0.30 M NaCl
B
2.90 M NaCl
ATCase specific activity as μM carbamyl aspartate/min/mg protein
% maximum ATCase activity
NaCl concentration, M
0
50
100
150
200
250
25
75
0.1
0.2
0.3
1.0
2.0
3.0
4.0

BIBLIOGRAPHY

Abram, D., and N. E. Gibbons. 1960. Turbidity of suspensions and morphology of red halophilic bacteria as influenced by sodium chloride concentration. Can. J. Microbiol. 6:535-543.

Abram, D., and N. E. Gibbons. 1961. The effect of chlorides of monovalent cations, urea, detergents, and heat on morphology and the turbidity of suspensions of red halophilic bacteria. Can. J. Microbiol. 7:741-750.

Abramson, D., and M. Belcher. 1964. Quantitative two-dimensional thin-layer chromatography of naturally occurring phospholipids. J. Lipid Res. 5:628-631.

Alexander, M. 1971. Microbial ecology. John Wiley and Sons, New York. 511 p.

Altman, P. L., and D. S. Dittmer (ed.). 1972. Biology data handbook, 2nd ed., vol. I, p. 471-742. Fed. Amer. Soc. Exptl. Biol., Bethesda.

Anderson, H. 1954. The reddening of salted hides and fish. Appl. Microbiol. 2:64-69.

Allen, R. J. L. 1940. The estimation of phosphorus. Biochem. J. 34:858-865.

Asbell, M. A., and R. G. Eagon. 1966. Role of multivalent cations in the organization, structure, and assembly of the cell wall of Pseudomonas aeruginosa. J. Bacteriol. 92:380-387.

Baars, J. K. 1930. Over sulfaatreductic door bacteriën. Ph.D. Dissertation. T'nische Hooge-School, Delft, The Netherlands. 107 p.

Baas-Becking, L. G. M. 1931. Historical notes on salt and salt manufacture. Sci. Monthly. 32:434-446.

Baird-Parker, A. C. 1963. A classification of micrococci and staphylocci based on physiological and biochemical tests. J. Gen. Microbiol. 30:409-427.

Batterton, J. C., Jr., and C. Van Baalen. 1971. Growth responses of blue-green algae to sodium chloride concentrations. Arch. Mikrobiol. 76:151-165.

Baumann, L., P. Baumann, M. Mandel, and R. E. Allen. 1972. Taxonomy of aerobic marine eubacteria. J. Bacteriol. 110:402-429.

Baxter, R. M. 1959. An interpretation of the effects of salts on the lactic dehydrogenase of Halobacterium salinarium. Can. J. Microbiol. 5:47-57.

Baxter, R. M., and N. E. Gibbons. 1954. The glycerol dehydrogenases of Pseudomonas salinaria, Vibrio costicolus, and Escherichia coli in relation to bacterial halophilism. Can. J. Biochem. Physiol. 32:306-217.

Baxter, R. M., and N. E. Gibbons. 1956. Effects of sodium and potassium chloride on certain enzymes of Micrococcus halodenitrificans and Pseudomonas salinaria. Can. J. Microbiol. 2:599-606.

Baxter, R. M., and N. E. Gibbons. 1957. The cysteine desulphydrase of Pseudomonas salinaria. Can. J. Microbiol. 3:461-465.

Bell, R. G., and D. J. Latham. 1975. Influence of NaCl, Ca, and Mg on the growth of a marine Bdellovibrio sp. Est. Coastal Mar. Sci. 3:381-384.

Ben-Amotz, A., and M. Avron . 1973. The role of glycerol in the osmotic regulation of the halophilic alga Dunaliella parva. Plant Physiol. 51:875-878.

Blankenship, L. C.,and R. N. Doetsch. 1961. Influence of a bacterial cell extract upon the morphogenesis of Arthrobacter ureafaciens. J. Bacteriol. 82:882-888.

Borowitzka, L. J., and A. D. Brown. 1974. The salt relations of marine and halophilic species of the unicellular green alga, Dunaliella. The role of glycerol as a compatible solute. Arch. Microbiol. 96:37-52.

Brock, T. D. 1969. Microbial growth under extreme conditions, p. 15-43. In P. Meadow and S. J. Pirt (ed.), Microbial growth, vol. XIX. Sym-Soc. Gen. Microbiol. Cambridge Univ. Press, Cambridge, U.K.

Brock, T. D. 1970. Principles of microbial ecology. Prentice-Hall, New Jersey. 306 p.

Broers, A. N., B. J. Panessa, and J. S. Gennaro, Jr. 1975. High resolution SEM of biological specimens, p. 234-242. In O. Johari and I. Corvin (ed.), Scanning electron microscopy, proceedings of the 8th annual scanning electron microscopy symposium, p. 234-242. SEM Symposia Office, Chicago.

Brown, A. D. 1960. Some properties of a gram-negative heterotrophic marine bacterium. J. Gen. Microbiol. 23:471-485.

Brown, A. D. 1961. Effects of salt concentration during growth on properties of the cell envelope of a marine pseudomonad. Biochim. Biophys. Acta 49:585-588.

Brown, A. D. 1962. The peripheral structures of gram-negative bacteria. III. Effects of cations on proteolytic degradation of the cell envelope of a marine pseudomonad. Biochim. Biophys. Acta 62:132-144.

Brown, A. D. 1963. The peripheral structures of gram-negative bacteria. IV. The cation-sensitive dissolution of the cell membrane of the halophilic bacterium, Halobacterium halobium. Biochim. Biophys. Acta 75:425-435.

Brown, A. D. 1964. Aspects of bacterial response to the ionic environment. Bacteriol. Rev. 28:296-329.

Brown, A. D. 1965. Hydrogen ion titrations of intact and dissolved liproprotein membranes. J. Mol. Biol. 12:491-508.

Brown, A. D., and N. E. Gibbons. 1955. The effect of magnesium, potassium, and iron on the growth and morphology of red halophilic bacteria. Can. J. Microbiol. 1:486-494.

Brown, A. D., and C. D. Shorey. 1963. The cell envelopes of two extremely halophilic bacteria. J. Cell Biol. 18:681-689.

Brown, A. D., and J. R. Simpson. 1972. Water relations of sugar-tolerant yeasts: the role of intracellular polyols. J. Gen. Microbiol. 72:589-591.

Brown, A. D., and H. P. Turner. 1963. Membrane stability and salt tolerance in gram-negative bacteria. Nature 199:301-302.

Bryant, M. P., I. M. Robinson, and H. Chu. 1959. Observations on the nutrition of Bacteroides succinogenes - a ruminal cellulytic bacterium. J. Dairy Sci. 42:1831-1847.

Buchanan, R. E., and N. E. Gibbons (ed.). 1974. Bergey's Manual of Determinative Bacteriology, 8th ed. Williams and Wilkens, Baltimore. 1268 p.

Buckmire, F. L. A., and R. A. MacLeod. 1965. Nutrition and metabolism of marine bacteria. XIV. On the mechanism of lysis of marine bacterium. Can. J. Microbiol. 11:677-691.

Buckmire, F. L. A., and R. A. MacLeod. 1971. Evidence for the release at low salt concentration of a lipid-protein-carbohydrate complex from isolated envelopes and whole cells of a marine pseudomonad. Can. J. Microbiol. 17:713-723.

Burick, E. 1950. Über die beziehungen zwischen hydratur und wachstum bei bakterien und hefen. Arch. Mikrobiol. 15:203-235.

Burton, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315-322.

Cessi, C., and F. Piliego. 1960. The determination of amino sugars in the presence of amino acids and glucose. Biochem. J. 77:508-510.

Chance, B. 1954. Spectrophotometry of intracellular respiratory pigments. Science 120:767-776.

Christian, J. H. B. 1955. The water relations of growth and respiration of Salmonella oranienburg at 30 C. Aust. J. Biol. Sci. 8:490-497.

Christian, J. H. B. 1956. The physiological basis of salt tolerance in halophilic bacteria. Ph.D. Thesis. Univ. Cambridge. 97 p.

Christian, J. H. B., and M. Ingram. 1959a. The freezing point of bacterial cells in relation to halophilism. J. Gen. Microbiol. 20:27-31.

Christian, J. H. B., and M. Ingram. 1959b. Lysis of Vibrio costicolus by osmotic shock. J. Gen. Microbiol. 20:32-42.

Christian, J. H. B., and J. A. Waltho. 1961. The sodium and potassium content of nonhalophilic bacteria in relation to salt tolerance. J. Gen. Microbiol. 25:97-102.

Christian, J. H. B., and J. A. Waltho. 1962. Solute concentrations within cells of halophilic and nonhalophilic bacteria. Biochim. Biophys. Acta 65:506-508.

Citarella, R. V., and R. R. Colwell. 1970. Polyphasic taxonomy of the genus Vibrio: polynucleotide sequence relationships among selected Vibrio species. J. Bacteriol. 104:434-442.

Cole, G. A., and R. J. Brown. 1967. The chemistry of Artemia habitats. Ecology 48:858-861.

Colwell, R. R., and W. J. Wiebe. 1970. "Core" characteristics for use in classifying aerobic, heterotrophic bacteria by numerical taxonomy. Bull. Georgia Acad. Sci. 28:165-185.

Costerton, J. W., C. Forsberg, T. I. Matula, F. L. A. Buckmire, and R. A. MacLeod. 1967. Nutrition and metabolism of marine bacteria. XVI. Formation of protoplasts, spheroplasts, and related forms from a gram-negative marine bacterium. J. Bacteriol. 94: 1764-1777.

Costerton, J. W., J. M. Ingram, and K. J. Cheng. 1974. Structure and function of the cell envelope of gram-negative bacteria. Bacteriol. Rev. 38:87-110.

Crane, R. K. 1965. Na-dependent transport in the intestine and other animal tissue. Fedn. Proc. Fedn. Am. Socs. Exp. Biol. 24:1000-1006.

D'Aoust, J. Y., and D. J. Kushner. 1971. Structural changes during lysis of a psychrophilic marine bacterium. J. Bacteriol. 108:916-927.

Devoe, I. W., and E. L. Oginsky. 1969a. Antagonistic effect of monovalent cations in maintenance of cellular integrity of a marine bacterium. J. Bacteriol. 98:1355-1367.

Devoe, I. W., and E. L. Oginsky. 1969b. Cation interactions and biochemical composition of the cell envelope of a marine bacterium. J. Bacteriol. 98:1368-1377.

Devoe, I. W., J. Thompson, J. W. Costerton, and R. A. MacLeod. 1970. Stability and comparative transport capacity of cells, mureinoplasts, and true protoplasts of a gram-negative bacterium. J. Bacteriol. 101:1014-1026.

Doetsch, R. N., and T. M. Cook. 1973. Introduction to bacteria and their ecobiology. Univ. Park Press, Baltimore. 371 p.

Dolin, M. I. 1961. Survey of microbial electron transport mechanisms, p. 319-364. In I. C. Gunsalus and R. Y. Stanier (ed.), The Bacteria: A treatise on structure and function, vol. II. Academic Press Inc., New York.

Doudoroff, M. 1940. Experiments on the adaptation of Escherichia coli to sodium chloride. J. Gen. Physiol. 23:585-611.

Drapeau, G. R., and R. A. MacLeod. 1963. Na-dependent active transport of aminoisobutyric acid into the cells of a marine pseudomonad. Biochem. Biophys. Res. Common. 12:111-115.

Drapeau, G. R., and R. A. MacLeod. 1965. A role for inorganic ions in the maintenance of intracellular solute concentrations in a marine pseudomonad. Nature 206:531.

Drapeau, G. R., T. I. Matula, and R. A. MacLeod. 1966. Nutrition and metabolism of marine bacteria. XV. Relation of Na-activated transport to the Na requirement of a marine pseudomonad for growth. J. Bacteriol. 92:63-71.

Dubos, R., and B. F. Miller. 1937. The production of bacterial enzymes capable of decomposing creatinine. J. Biol. Chem. 121:429-445.

Dussault, H. P. 1933. Effect of prolonged storage on the fate of halophilic bacteria in contaminated salt. Fish. Res. Bd. Can. Prog. Repts. Atlantic Coast Sts. No. 55: 7-9.

Dussault, H. P. 1955. An improved technique for staining red halophilic bacteria. J. Bacteriol. 70:484-485.

Eagon, R. G. 1969. Cell wall-associated inorganic substances from Pseudomonas aeruginosa. Can. J. Microbiol. 15:135-139.

Eigen, M., and R. G. Wilkens. 1965. The kinetics and mechanism of formation of metal complexes. Adv. Chem. Ser. 49:55-67.

Elazari-Volcani, B. 1940. Studies on the microflora of the Dead Sea. Ph.D. Thesis. Hebrew Univ., Jerusalem. 120 p.

Elazari-Volcani, B. 1943. A dimastigamoeba in the bed of the Dead Sea. Nature 152:301-302.

Elazari-Volcani, B. 1944. A ciliate from the Dead Sea. Nature 154:335.

Fischer, B. 1894. Die bakterien des meeres nach den untersuchungen der plankton-expedition unter gleichzeitiger berücksichtigung einiger alterer und neueres untersuchungen. Ergebnisse der Plankton-Expedition der Humbolt-Stiftung. Verlag vonLipsius und Tischer, Kiel und Leipzig, Germany 4:1-81.

Fiske, C., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66:375-400.

Flannery, W. L. 1956. Current status of knowledge of halophilic bacteria. Bacteriol. Rev. 20:49-66.

Flannery, W. L., R. N. Doetsch, and P. A. Hansen. 1953. Salt desideratum of Vibrio costicolus, an obligate halophilic bacterium. II. Effect of salts on the oxidation of glucose. J. Bacteriol. 66:526-530.

Flowers, S., and F. R. Evans. 1966. The flora and fauna of the Great Salt Lake Region, Utah, p. 367-393. In H. Boyko (ed.), Salinity and Aridity. Dr. W. Junk Publisher, The Hague.

Foda, I. O., and R. H. Vaughn. 1950. Salt tolerance in the genus Aerobacter. Food Tech., Champaign 4:182-188.

Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.

Forsberg, C. W., J. Costerton, and R. A. MacLeod. 1970a. Separation and localization of cell wall layers of a gram-negative bacterium. J. Bacteriol. 104:1338-1353.

Forsberg, C. W., J. Costerton, and R. A. MacLeod. 1970b. Quantitation, chemical characteristics, and ultrastructure of the three outer cell wall layers of a gram-negative bacterium. J. Bacteriol. 104:1354-1368.

Forsberg, C. W., M. K. Rayman, J. W. Costerton, and R. A. MacLeod. 1972. Isolation, characterization, and ultrastructure of the peptidoglycan layer of marine pseudomonad. J. Bacteriol. 109:895-905.

Forsyth, M. P., and D. J. Kushner. 1970. Nutrition and distribution of salt response in populations of moderately halophilic bacteria. Can. J. Microbiol. 16:253-261.

Forsyth, M. P., D. B. Shindler, M. B. Gochnauer, and D. J. Kushner. 1971. Salt tolerance of intertidal marine bacteria. Can. J. Microbiol. 17:825-828.

Frank, M., and E. Hess. 1941a. Studies on salt fish. V. Studies on Sporendonema epizoum from "dun" salt fish. J. Fish. Res. Bd. Can. 5:276-286.

Frank, M., and E. Hess. 1941b. Studied on salt fish. VI. Halophilic brown molds of the genus Sporendonema emend. Ciferri et Redaelli. J. Fish. Res. Bd. Can. 5:287-292.

Gibbons, N. E., and R. M. Baxter. 1953. The relation between salt concentration and enzyme activity in halophilic bacteria. Proc. VIth Int. Congr. Microbiol., Rome 1:210-214.

Gibor, A. 1956. The culture of brine algae. Biol. Bull. 111:223-229.

Ginzburg, M., L. Sachs, and B. Z. Ginzburg. 1970. Ion metabolism in a Halobacterium. I. Influence of age of culture on intracellular concentrations. J. Gen. Physiol. 55:187-207.

Ginzburg, M., L. Sachs, and B. Z. Ginzburg. 1971. Ion metabolism in a Halobacterium. II. Ion concentrations in cells at different levels of metabolism. J. Membrane Biol. 5:78-101.

Gochnauer, M. B., and D. J. Kushner. 1971. Potassium binding, growth, and survival of an extremely halophilic bacterium. Can. J. Microbiol. 17:17-23.

Gochnauer, M. B., G. G. Leppard, P. Komaratat, M. Kates, T. Novitsky, and D. J. Kushner. 1975. Isolation and characterization of Actinopolyspora halophila, gen. et sp. nov., an extremely halophilic actinomycete. Can. J. Microbiol. 21:1500-1511.

Goldman, M., R. H. Deibel, and C. F. Niven, Jr. 1963. Interrelations between temperature and sodium chloride on growth of lactic acid bacteria isolated from meat curing brines. J. Bacteriol. 85:1017-1021.

Harvey, E. N. 1915. The effect of certain organic and inorganic substances upon light production by luminous bacteria. Biol. Bull. 29:308-311.

Hayat, M. A. 1970. Principles and techniques of electron microscopy, vol. 1. Van Nostr. and Reinhold, New York. 412 p.

Hess, E. 1942. Studies on salt fish. IX. Effect of environment upon the growth of red halophilic bacteria. J. Fish. Res. Bd. Can. 6:10-16.

Hidaka, T., and M. Sakai. 1970. Comparative observation of the inorganic salt requirements of the marine and terrestrial bacteria. Bull. Misaki Mar. Biol. Fish. 36:125-149.

Hill, R. 1965. Hydrolysis of proteins. Adv. Prot. Chem. 20:37-107.

Hill, S. E. 1929. The penetration of luminous bacteria by the ammonium salts of fatty acids. Part I. General outline of the problem and the effects of strong acids and alkalies. J. Gen. Physiol. 12:863-872.

Hill, S. E., and E. C. White. 1929. Sodium chloride media for the separation of certain gram-positive cocci from gram-negative bacilli. J. Bacteriol. 18:43-57.

Hof, T. 1935. Investigations concerning bacterial life in strong brines. Recueil des Trav. Bot. Neerl. 32:92-173.

Hodgman, C. D. (ed.). 1959. Handbook of chemistry and physics, 40th ed. p. 652-653. Chemical Rubber Publishers, Cleveland.

Holmes, P. K. 1964. Studies of a halophilic enzyme. Ph.D. Dissertation. Univ. Illinois. 133 p.

Ingram, M. 1947. A theory relating the action of salts on bacterial respiration to their influence on the solubility of proteins. Proc. Roy. Soc. B. 134:181-201.

Ingram, M. 1957. Microorganisms resisting high concentrations of sugars or salts, p. 90-133. In R. E. O. Williams and C. C. Spier (ed.), Microbial ecology. Cambridge Univ. Press, Cambridge, U.K.

Ishida, Y. and T. Fujii. 1970. Isolation of halophilic and halotolerant bacteria from solar salt. Bull. Jap. Soc. Sci. Fish. 36:391-401.

Jacobs, S. 1965. The determination of nitrogen in biological materials. Meth. Biochem. Anal. 13:241-263.

Johnson, M. K., E. J. Johnson, R. D. MacElroy, H. L. Speer, and B. S. Bruff. 1968. Effects of salts on the halophilic alga Dunaliella viridis. J. Bacteriol. 95:1461-1468.

Kaplan, J. G., M. Duphil, and F. LaCroute. 1967. A study of the aspartate transcarbamylase activity of yeast. Arch. Biochem. Biophys. 119:541-551.

Kates, M., P. S. Sastry, and L. S. Yengoyan. 1963. Isolation and characterization of a diether analog of phosphatidylglycerophosphate from Halobacterium cutirubrum. Biochim. Biophys. Acta 70:705-707.

Kates, M., L. S. Yengoyan, and P. S. Sastry. 1965. A diether analog of phosphatidylglycerophosphate in Halobacterium cutirubrum. Biochim. Biophys. Acta 98:252-268.

Keller, P., and Y. Henis. 1967. The effect of yeast extract and casitone on the response to salt of the microflora of a highly saline soil. Can. J. Microbiol. 13:1427-1432.

Kipnis, D. M., and J. E. Parrish. 1965. Role of Na and K on sugar (2-deoxyglucose) and amino acid (α-aminoisobutyric acid) transport in striated muscle. Fedn. Proc. Fedn. Am. Socs. Exp. Biol. 24:1051-1059.

King, E.O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein J. Lab. Clin. Med. 44:301-307.

Kleinkeller, A., and A. Kotyk (ed .). 1961. Membrane transport and metabolism. Academic Press, New York. 608 p.

Kluyver, A. J., and J. K. Baars. 1932. On some physiological artefacts. Proc. Acad. Sci. Amst. 35:370-378.

Knudsen, M. 1901. Hydrographical tables. G. E. C. Gad, Copenhagen. 63 p.

Kocur, M., and W. Hodgkiss. 1973. Taxonomic status of the genus Halococcus Schoop. Int. J. Syst. Bacteriol. 23:151-156.

Koritz, S. B., and P. P. Cohen. 1954. Colorimetric determination of carbamylamino acids and related compounds. J. Biol. Chem. 209:145-150.

Kotin, J. 1963. On the effect of ionic strength on the melting temperature of DNA. J. Mol. Biol. 7:309-311.

Kushner, D. J. 1964. Lysis and dissolution of cells and envelopes of an extremely halophilic bacterium. J. Bacteriol. 87:1147-1156.

Kushner, D. J. 1968. Halophilic bacteria. Adv. Appl. Microbiol. 10:73-99.

Kushner, D. J., S. T. Bayley, J. Boring, M. Kates, and N. E. Gibbons. 1964. Morphological and chemical properties of cell envelopes of the extreme halophile, Halobacterium cutirubrum. Can. J. Microbiol. 10:483-497.

Kushner, D. J., and H. Onishi. 1966. Contribution of protein and lipid components to the salt response of envelopes of an extremely halophilic bacterium. J. Bacteriol. 91:653-660.

Laemmeli, U. K. 1970. Cleavage of structural proteins durin the assembly of the head of bacteriophage T_4. Nature 227:680-685.

Lamanna, C.,and M. F. Mallette. 1965. Basic Bacteriology, 3rd ed., p. 333-384. Williams and Wilkins, Baltimore.

Lanyi, J. K., and M. P. Silverman. 1972. The state of binding of intracellular K on Halobacterium cutirubrum. Can. J. Microbiol. 18:993-995.

Lapage, S. P., P. H. A. Sneath, E. F. Lessel, V. B. D. Skerman, H. P. R. Seeliger, and W. A. Clark (ed.). 1975. International Code of Nomenclature of Bacteria. Amer. Soc. Microbiol., Washington, D. C. 180 p.

Lardy, H. A. 1949. Respiratory enzymes. Burgess Publish., Minn. 290 p.

Larsen, H. 1962. Halophilism, p. 297-342. In I. C. Gunsalus and R. Y. Stanier (ed.), The Bacteria: A treatise on Structure and Function, vol. IV. Academic Press, Inc., New York.

Larsen, H. 1967. Biochemical aspects of extreme halophilism. Adv. Microbial Physiol. 1:97-132.

Larsen, H. 1973. The halobacteria's confusion to biology. Antonie van Leeuwenhoek, J. Microbiol. Serol. 39:383-396.

Lederberg, J., and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63:399-406.

Lehninger, A. L. 1975. Biochemistry, 2nd ed. Worth Publishers, Inc., New York. 1104 p.

Liebl, V., J. G. Kaplan, and D. S. Kushner. 1969. Regulation of a salt-dependent enzyme: the aspartate transcarbamylase of an extreme halophile. Can. J. Biochem. 47:1095-1097.

Limsong, S., and W. C. Frazier. 1966. Adaptation of Pseudomonas fluorescens to low levels of water activity produced by different solutes. Appl. Microbiol. 14:899-901.

Lindeberg, G. 1958. Laevan-forming halophilic bacteria. Nature 180:1141.

Lindegren, C. C., S. Nagai, and H. Nagai. 1958. Induction of respiratory deficiency in yeast by manganese, copper, cobalt, and nickel. Nature 182:446-448.

Littlewood, D., and J. R. Postgate. 1957. Sodium chloride and the growth of Desulphovibrio desulfuricans. J. Gen. Microbiol. 17:378-389.

Loefer, J. 1939. Acclimation of fresh water ciliates and flagellates to media of higher osmotic pressure. Physiol. Zool. 12:161-172.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin-phenol reagent. J. Biol. Chem. 193:265-275.

Lyman, J., and R. H. Fleming. 1940. Composition of seawater. J. Mar. Res. 3:134-146.

MacLeod, R. A. 1965. The question of the existence of specific marine bacteria. Bacteriol. Rev. 29:9-23.

MacLeod, R. A. 1968. On the role of inorganic ions in the physiology of marine bacteria, p. 95-126. In M. R. Droop and E. J. Ferguson Wood (ed.), Advances in Microbiology of the sea, vol. 1. Academic Press, Inc., New York.

MacLeod, R. A., C. A. Claridge, A. Hori, and J. F. Murray. 1958. Observations on the function of sodium in the metabolism of marine bacteria. J. Biol. Chem. 232:829-834.

MacLeod, R. A., and A. Hori. 1960. Nutrition and metabolism of marine bacteria. VIII. Tricarboxylic acid cycle enzymes in a marine bacterium and their response to added salts. J. Bacteriol. 80:464-471.

MacLeod, R. A., A. Hori, and S. M. Fox. 1960. Nutrition and metabolism of marine bacteria. IX. Ion requirements for obtaining and stabilizing isocitric dehydrogenase from a marine bacterium. Can. J. Biochem. Physiol. 38:693-701.

MacLeod, R. A., and T. I. Matula. 1961. Solute requirements for preventing lysis of some marine bacteria. Nature (London) 192:1209-1210.

MacLeod, R. A., and T. I. Matula. 1962. Nutrition and metabolism of marine bacteria. XI. Some characteristics of the lytic phenomenon. Can. J. Microbiol. 8:883-896.

MacLeod, R. A., and E. Onofrey. 1956. Nutrition and metabolism of marine bacteria. II. Observations on the relation of seawater to the growth of marine bacteria. J. Bacteriol. 71:661-667.

MacLeod, R. A., and E. Onofrey. 1957a. Nutrition and metabolism of marine bacteria. III. The relation of sodium and potassium to growth. J. Cell. Comp. Physiol. 50:389-402.

MacLeod, R.A., and E. Onofrey. 1957b. Nutrition and metabolism of marine bacteria. VI. Quantitative requirements for halides, magnesium, calcium, and iron. Can. J. Microbiol. 3:753-759.

MacLeod, R.A., and E. Onofrey. 1958. Nutrition and metabolism of marine bacteria. VII. Growth response of a marine Flavobacterium to surface-active agents and nucleotides. J. Bacteriol. 75:460-466.

MacLeod, R.A., and E. Onofrey. 1963. Studies on the stability of the Na requirement of marine bacteria. In C.H. Oppenheimer (ed.), Symposium on Marine Microbiology, pp. 481-489. Charles C. Thomas, Springfield, Illinois.

Mager, J. 1959a. The stabilizing effect of spermine and related polyamines and bacterial protoplasts. Biochim. Biophys. Acta 36:529-531.

Mager, J. 1959b. Spermine as a protective agent against osmotic lysis. Nature 183:1827-1828.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3:208-218.

Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5:109-118.

Marshall, C.L., A.J. Wicken, and A.D. Brown. 1969. The outer layer of the cell envelope of Halobacterium halobium. Can. J. Biochem. 47:71-74.

Masui, M., and S. Wada. 1973. Intracellular concentrations of Na, K, and Cl of a moderately halophilic bacterium. Can. J. Microbiol. 19:1181-1186.

Matheson, A.T., G. Dennis Sprott, I. J. McDonald, and H. Tessier. 1976. Some properties of an unidentified halophile: growth characteristics, internal salt concentration, and morphology. Can. J. Microbiol. 22:780-786.

Matula, T.I., V.S. Srivastava, P. Wong, and R.A. MacLeod. 1970. Transport and retention of K and other metabolites in a marine pseudomonad and their relation to the mechanism of optical effects. J. Bacteriol. 102:790-796.

Mayers, S.P., B. Prindle, and E.S. Reynolds. 1960. Cellulolytic activity of marine fungi. Degradation of lignocellulose material. Tappi 43:534-538.

McClare, C.W.F. 1967. Bonding between proteins and lipids in the envelopes of Halobacterium halobium. Nature 216:766-771.

Mejbaum, W. Z. 1939. Über die bestimmung kleiner pentosemengen insbesondere in derivaten der adenylsäure. Hoppe-Seyler's Z. Physiol. Chem. 258:117-120.

Menzel, D. W., and R. F. Vaccaro. 1964. The measurement of dissolved organic and particulate carbon in seawater. Limnol. and Oceanogr. 9:138-142.

Mescher, M. F., J. L. Strominger, and S. W. Watson. 1974. Protein and carbohydrate composition of the cell envelope of Halobacterium salinarium. J. Bacteriol. 120:945-954.

Meynell, G. G., and E. Meynell. 1970. Theory and practice in experimental bacteriology, 2nd ed. Cambridge Univ. Press, Cambridge. 347 p.

Miller, J. H. 1972. Experiments in molecular genetics. p. 338-343. Cold Spring Harbor, New York.

Mitchell, P.,and J. Moyle. 1956. Osmotic function and structure in bacteria, p. 150-180. In E. T. C. Spooner and B. A. D. Stocker (ed.), Bacterial anatomy, Sixth Symp. Soc. Gen. Microbiol. Cambridge Univ. Press, London.

Mohr, V., and H. Larsen. 1963. On the structural transformations and lysis of Halobacterium salinarium in hypotonic and isotonic solutions. J. Gen. Microbiol. 31:267-280.

Novitsky, T. J., and D. J. Kushner. 1975. Influence of temperature and salt concentration on the growth of a facultative halophilic "Micrococcus" sp. Can. J. Microbiol. 21:107-110.

Onishi, H. 1963. Osmophilic yeast. Adv. Food Res. 12:53-94.

Onishi, H., and D. J. Kushner. 1966. Mechanism of dissolution of envelopes of the extreme halophile Halobacterium cutirubrum. J. Bacteriol. 91:646-652.

Oparin, A. I. 1962. Life--its nature, origin, and development, p. 155-200. Academic Press, New York.

Oppenheimer, C. H., and C. E. ZoBell. 1952. The growth and viability of sixty-three species of marine bacteria as influenced by hydrostatic pressure. J. Mar. Res. 11: 10-18.

Passow, H., A. Rothstein, and T. W. Clarkson. 1961. The general pharmacology of heavy metals. Pharmacol. Rev. 13:185-224.

Payne, W. J. 1958. Studies on bacterial utilization of uronic acids. III. Induction of oxidative enzymes in a marine isolate. J. Bacteriol. 76:301-307.

Payne, W. J. 1960. Effects of sodium and potassium ions on growth and substrate penetration of a marine pseudomonad. J. Bacteriol. 80:696-700.

Pratt, D. B. 1963. Specificity of the solute requirement by marine bacteria on primary isolation from seawater. Nature (London) 199:1309.

Pratt, D. B. 1974. Salt requirement for growth and function of marine bacteria, p. 3-15. In R. R. Colwell and R. V. Morin (ed.), Effect of the ocean environment on microbial activities. Univ. Park Press, Baltimore.

Pratt, D. B., and M. Austin. 1963. Osmotic regulation of the growth rate of four species of marine bacteria, p. 629-637. In C. H. Oppenheimer (ed.), Symposium on Marine Microbiology. Charles C. Thomas, Springfield.

Pratt, D. B., and F. C. Happold. 1960. Requirements for indole production by cells and extracts of a marine bacterium. J. Bacteriol. 80:232-236.

Pratt, D. B., and W. H. Riley. 1955. Lysis of a marine bacterium in salt solutions. Bact. Proc. 55:26.

Pratt, D. B., and G. Waddell. 1959. Adaptation of marine bacteria to media lacking sodium chloride. Nature (London) 183:1208-1209.

Petrowa, E. K. 1933. Mikrobiologie des kochsalzes. Arch. Mikrobiol. 4:326-347.

Pritchard, D. W. 1967. What is an estuary: physical viewpoint, p. 3-5. In G. H. Lauff (ed.), Estuaries, Publ. No. 83. Amer. Ass. Adv. Sci., Washington, D. C.

Rajagopalan, K. V.,and P. Handler. 1968. Metalloflavoproteins, p. 301-337. In T. P. Singer (ed.), Biological oxidations. John Wiley and Sons, New York.

Rayman, M. K., R. C. Gordon, and R. A. MacLeod. 1967. Isolation of a Mg phospholipid from Halobacterium cutirubrum. J. Bacteriol. 93:1465-1466.

Rayman, M. K., and R. A. MacLeod. 1975. Interaction of Mg with peptidoglycan and its relation to the prevention of lysis of a marine pseudomonad. J. Bacteriol. 122: 650-659.

Reistad, R. 1970. On the composition and nature of the bulk protein of extremely halophilic bacteria. Arch. Mikrobiol. 71:353-360.

Rhodes, M. E., and W. J. Payne. 1962. Further observations on the effects of cations on enzyme induction in marine bacteria. Antonie Van Leeuwenhoek. 28:121-128.

Rhodes, M. E., and W. J. Payne. 1967. Influence of cations on spheroplasts of marine bacteria functioning as osmometers. Appl. Microbiol. 15:537-542.

Richter, O. 1928. Natrium: Ein notwendiges nahrelement für eine marine mikroarophile leuchtbakterie. Anz. Ost. Akad. Wiss., Math.--Nature. Kl. 101:261-292.

Riley, J. P. and G. Skirrow. 1975. Chemical oceanography, vol. 1. Academic Press, Inc., New York. 606 p.

Robinson, J., and N. E. Gibbons. 1952. The effects of salts on the growth of Micrococcus halodenitrificans. Can. J. Bot. 30:147-154.

Robinson, J., N. E. Gibbons, and F. S. Thatcher. 1952. A mechanism of halophilism in Micrococcus halodenitrificans. J. Bacteriol. 64:69-77.

Ruinen, J. 1938. Notizen über salzflagellaten. II. Über die vergreitung der salt flagellaten. Arch. Protistenk. 90:210-216.

Ryter, A. and E. Kellenberger. 1958. Etude an microscope electronique de plasmas contenant de l'acide desoxyribonucleique. I. Les nucleoldes des bacteries en croissance active. Z. Naturforsch. 136:597-599.

Salton, M. R. J. 1953. Studies on the bacterial cell wall. IV. The composition of the cell walls of some gram-positive and gram-negative bacteria. Biochim. Biophys. Acta 10:512-523.

Salton, M. R. J. 1964. The bacterial cell wall, p. 8-9. Elsevier Publishing Co., New York.

Salton, M. R. J. 1967. Structure and function of bacterial cell membranes. Ann. Rev. Microbiol. 21:417-442.

Schildkraut,T. C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. 4:430-433.

Schoop, G. 1934. Salzbakterien. Dtsch. tierarztl. Wschr. 42:205-225.

Schoop, G. 1935. Obligat halophile Mikroben Centbl. Bakt. Abt. I. Orig. 134:14-19.

Schultz, S. G., and P. F. Curran. 1970. Coupled transport of sodium and organic solutes. Physiol. Rev. 50:637-718.

Schultz, S. G., and A. K. Solomon. 1961. Cation transport in Escherichia coli. I. Intracellular Na and K concentrations and net cation movement. J. Gen. Physiol. 45:355-369.

Scott, W. J. 1957. Water relations of food spoilage microorganisms. Adv. Food Res. 7:83-127.

Sehgal, S. N., M. Kates, and N. E. Gibbons. 1962. Lipids of Halobacterium cutirubrum. Can. J. Biochem. Physiol. 40:69-81.

Seifer, S., S. Dayton, B. Novic, and E. Muntwyler. 1950. The estimation of glycogen with the anthrone reagent. Arch. Biochem. 25:191-200.

Squoros, P. L. 1955. Microbial transformations of the tobacco alkaloids. I. Cultural and morphological characteristics of a nicotinophile. J. Bacteriol. 69:28-37.

Shah, V. H., and T. D. H. deSa. 1964. Studies on halotolerant and halophilic bacteria: Part I--Isolation and salt response. Indian J. Exp. Biol. 2:181-184.

Shewan, J. M. 1938. The reddening of salt fish. Rep. Fd. Invest. Bd. (London) p. 113.

Shewan, J. M., W. Hodgkiss, and J. Liston. 1954. A method for the rapid differentiation of certain nonpathogenic asporogenous bacilli. Nature 173:208-209.

Sillén, L. G. 1961. The physical chemistry of seawater, p. 549-581. In M. Sears (ed.), Oceanography. Amer. Assoc. Adv. Sci., Pub. #67, Washington, D.C.

Sistrom, W. R. 1960. A requirement for sodium in the growth of Rhodopseudomonas spheroides. J. Gen. Microbiol. 22:778-785.

Skerman, V. B. D. 1967. A guide to the identification of the genera of bacteria. 2nd ed. Williams and Wilkens Co., Baltimore. 303 p.

Smith, W. W., and C. E. ZoBell. 1937. Direct microscopic evidence of an autochthonous bacterial flora in Great Salt Lake. Ecology 18:453-458.

Smithies, W. R., N. E. Gibbons, and S. T. Bayley. 1955. The chemical composition of the cell and cell wall of some halophilic bacteria. Can. J. Microbiol. 1:605-613.

Soc. Amer. Bacteriol. 1957. Manual of microbiological methods. McGraw-Hill, New York. 315 p.

Soo-Hoo, T. S., and A. D. Brown. 1967. A basis of the specific sodium requirement for morphological integrity of Halobacterium halobium. Biochim. Biophys. Acta 135:164-166.

Stanier, R. V. 1941. Studies on marine agar-digesting bacteria. J. Bacteriol. 42:527-557.

Stanier, R. V., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: A taxonomic study. J. Gen. Microbiol. 43:159-271.

Steensland, H., and H. Larsen. 1969. A study of the cell envelope of the halobacteria. J. Gen. Microbiol. 55:325-336.

Stevenson, J. 1966. The specific requirement for sodium chloride for the active uptake of L-glutamate by Halobacterium salinarium. Biochem. J. 99:257-260.

Stoeckenius, W., and R. Rowen. 1967. A morphological study of Halobacterium halobium and its lysis in media of low salt concentration. J. Cell Biol. 34:365-393.

Stuart, L. S. 1938. Isolation of halophilic bacteria from soil, water, and dung. Food Res. 3:417-428.

Stuart, L. S., and L. H. James. 1938. The effect of Eh and sodium chloride concentration on the physiology of halophilic bacteria. J. Bacteriol. 35:381-396.

Sud, I. J., and M. E. Tyler. 1964. Cell-wall composition and osmotic fragility of selected marine bacteria. J. Bacteriol. 87:696-700.

Sverdrup, H. U., M. W. Johnson, and R. H. Fleming. 1942. The oceans. Prentice-Hall, New York. 1087 p.

Takacs, F. P., T. I. Matula, and R. A. MacLeod. 1964. Nutrition and metabolism of marine bacteria. XIII. Intracellular Na and K concentrations in a marine pseudomonad. J. Bacteriol. 87:510-518.

Takahashi, K., and N. E. Gibbons. 1959. Effect of salt concentration on the morphology and chemical composition of Micrococcus halodenitrificans. Can. J. Microbiol. 5:25-35.

Tedder, S. 1966. Replacement of sodium ions by potassium ion in the growth of selected marine organisms. M. S. Thesis. Univ. Florida, Gainesville. p. 153.

Thompson, J., and R. A. MacLeod. 1971. Function of Na and K in the active transport of α-aminoisobutyric acid in a marine pseudomonad. J. Biol. Chem. 246:4066-4074.

Thompson, J., and R. A. MacLeod. 1973. Na and K gradients and α-aminoisobutyric acid transport in a marine pseudomonad. J. Biol. Chem. 248:7106-7111.

Thornley, M. J. 1960. The differentiation of Pseudomonas from other gram-negative bacteria on the basis of arginine metabolism. J. Appl. Bacteriol. 23:35-52.

Tomlinson, N., and R. A. MacLeod. 1957. Nutrition and metabolism of marine bacteria. IV. The participation of Na, K, and Mg salts in the oxidation of exogenous substrates by a marine bacterium. Can. J. Microbiol. 3:627-638.

Tresner, H. D., J. A. Hayes, and E. J. Backus. 1968. Differential tolerance of Streptomycetes to sodium chloride as a taxonomy aid. Appl. Microbiol. 16:1134-1136.

Tyler, M. E., M. C. Bielling, and D. B. Pratt. 1960. Mineral requirements and other characters of selected marine bacteria. J. Gen. Microbiol. 23:153-161.

Ueno, T. 1964. Studies on halophilic bacteria in soy sauce. Bull. Univ. Osaka Pref., Ser. B. 15:67-113.

Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. Manometric techniques, 4th ed. Burgess Publishers, Minneapolis. 305 p.

Unemoto, T., T. Tsuruoka, and M. Hayashi. 1973. Role of Na and K in preventing lysis of a slightly halophilic Vibrio alginolyticus. Can. J. Microbiol. 19:563-571.

Vaisey, E. B. 1954. Osmophilism of Sporendonema epizoum. J. Fish. Res. Bd. Can. 11:901-903.

Vaituzis, Z., and R. N. Doetsch. 1966. Flagella of Escherichia coli spheroplasts. J. Bacteriol. 91:2103-2104.

Vaituzis, Z., and R. N. Doetsch. 1969. Relationship between cell wall, cytoplasmic membrane, and bacterial motility. J. Bacteriol. 100:512-521.

Watson, T. G. 1970. Effects of sodium chloride on steady-state growth and metabolism of Saccharomyces cerevisiae. J. Gen. Microbiol. 64:91-99.

Weast, R. C. (ed.). 1974. Handbook of chemistry and physics, 54th ed., p. D-203, D-204, D-222, D-223, and D-229. Chemical Rubber Publishers, Cleveland.

Weber, M. M. 1949. Red halophilic micro-organisms. A problem on the biochemical foundations of ecological specialization. Biol. Rev. City College, New York 11:9-14.

West, N. S., J. R. Gililland, and R. H. Vaughn. 1941. Characteristics of coliform bacteria from olives. J. Bacteriol. 41:341-353.

Wong, P. T. S., J. Thompson, and R. A. MacLeod. 1969. Nutrition and metabolism of marine bacteria. XVII. Iondependent retention of α-amino-isobutyric acid and its relation to Na-dependent transport in a marine pseudomonas. J. Biol. Chem. 244:1016-1025.

Yamada, T., and A. Asano. 1954. Oxidation reduction enzymes of a halophilic bacterium. J. Biochem., Tokyo. 41: 639-645.

Yamada, T., and I. Shiio. 1953. Effects of salt concentration on the respiration of a halotolerant bacterium. J. Biochem., Tokyo. 40:327-337.

Yoshii, H. 1967. Salt and microorganisms. Characteristics of halophilic bacteria. Nippon Jozo Kyokai Zasshi 62:43-49.

ZoBell, C. E. 1941. Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. J. Mar. Res. 4:42-71.

ZoBell, C. E. 1946. Marine microbiology. Chronica Botanica, Waltham, Mass. 240 p.

ZoBell, C. E., D. Q. Anderson, and W. W. Smith. 1937. The bacteriostatic and bactericidal action of Great Salt Lake water. J. Bacteriol. 33:253-262.

ZoBell, C. E., and H. D. Michener. 1938. A. paradox in the adaptation of marine bacteria to hypotonic solutions. Science 87:328-329.

ZoBell, C. E., and H. C. Upham. 1944. A list of marine bacteria including descriptions of sixty new species. Bull. Scripps. Inst. Oceanogr. 5(2):239-292.